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FINAL REPORT

No. 1105-F

TAPE CASSETTE BACTERIA DETECTION SYSTEM

To

NASA/MANNED SPACECRAFT CENTER

Houston, Texas 77058

Contract NAS 9-12548

27 April 1973

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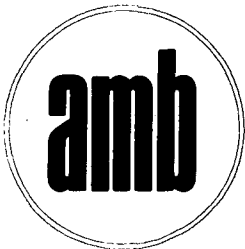
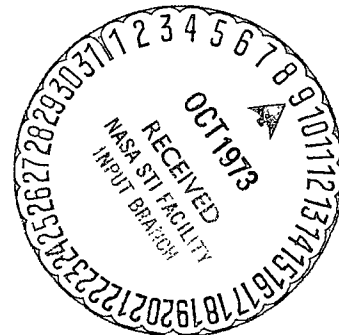
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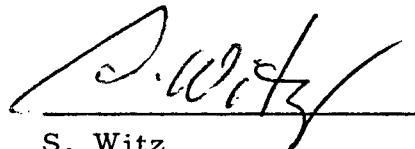
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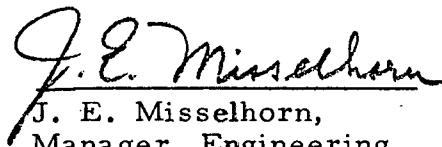
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Houston, Texas 77058

Contract NAS 9-12548

27 April 1973



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CONTENTS

<u>Section</u>		<u>Page</u>
1	SUMMARY	1
2	INTRODUCTION	3
3	EXPERIMENTAL RESULTS	4
3.1	Instrumentation	4
3.2	Instrument Reliability	22
3.3	Unincubated Cycle	26
3.4	Incubated Cycle	36
3.5	Operation in an Automatic Mode	36
3.6	Operation in a Simulated Zero-g Environment	40
3.7	Demonstration of Instrument	40
4	LABORATORY SUPPORT STUDIES	45
4.1	Establishing Design Parameters of Prototype Test Capsule	45
4.2	Studies with Capsule Prototype	54
4.3	Finalized Capsule Design	63
4.4	Capsule Pretreatment	63
4.5	Cross-Contamination Study	70
4.6	Reagent Shelf Life	71
4.7	Effect of Anode Voltage on PMT Output	75
5	CONCLUSIONS AND RECOMMENDATIONS	78

APPENDICES

<u>Appendix</u>		<u>Page</u>
A	STARTUP PROCEDURE	81
B	MANUAL OPERATIONAL PROCEDURE	83
C	SHUTDOWN PROCEDURE	86
D	REAGENT PREPARATION	87

TABLES

<u>Table</u>	<u>Page</u>
1 Capsule Assembly	6
2 Operational Sequence for Processing Station	19
3 Processing Sequence	23
4 AMB Water Monitor Specifications	24
5 Signal Response - Unincubated Cycle	34
6 Signal Response - Unincubated Cycle (Stopped-Flow - Dry)	35
7 Signal Response - Incubated Cycle	37
8 Automatic Program Sequence (Stepper #1)	38
9 Automatic Program Sequence (Stepper #2)	39
10 Demonstration of AMB Water Monitor to DCAS Representatives	42
11 Test Results of Instrument Demonstration	43
12 Effect of Pressure Filtration on Various Filter Combinations	52
13 Chemiluminescence of Water Extractables from Membrane Filter-Absorbent Pad Combination	53
14 Chemiluminescence Study with Filter-Screen Combination in Prototype Capsule	55
15 Leakage Studies - Test Capsule	60
16 Effect of Dry/Wet Capsule Filter on Chemiluminescence Signal	62
17 Chemiluminescence Signal of Unincubated Water Samples Containing <u>E. coli</u>	64
18 Chemiluminescence Signal of Incubated Water Samples Containing <u>E. coli</u>	66
19 Capsule Pretreatment	69
20 Effect of Aging Luminol and H ₂ O ₂ on Chemiluminescence Signal	72
21 Effect of Aging Urea on Chemiluminescence Signal	74
22 Anode Voltage Vs PMT Output	76

ILLUSTRATIONS

<u>Figure</u>		<u>Page</u>
1	Exploded View - Capsule	5
2	Capsule Mounted on Transport Film	7
3	Mechanical Hand Punch	9
4	Tape Transport Bacteria Detection System	10
5	Electronic Control Chassis	11
6	Control Panel	12
7	Pump and Valve Assembly	13
8	Processing Station Assembly	14
9	Processing Station Assembly (Light Shield Removed) . . .	15
10	Processing Station Assembly (Back Panel)	16
11	Processing Station (Schematic)	18
12	PMT Housing Assembly	20
13	Liquid Flow Schematic	21
14	PMT Housing Assembly and Light Shield	27
15	Reagent Flow in Capsule	28
16	Oscilloscope Traces	30
17	Signal Intensity Vs. Time - <u>E. coli</u>	31
18	Signal Intensity Vs. Time - <u>S. marcescens</u>	32
19	AMB Water Monitor Operated in Inverted Position (180°)	41
20	Test Capsule (Schematic)	46
21	Bubble Point Apparatus	50
22	Test Capsule (Bottom View)	56
23	Body, Test Capsule	57
24	Top Plate, Test Capsule	58
25	Bottom Plate, Test Capsule	59
26	Signal Response of <u>E. coli</u>	65
27	Cap, Window	67
28	Body, Capsule	68
29	Anode Voltage Vs. PMT Output	77

Section 1

SUMMARY

The design, fabrication, and testing of an automatic bacteria detection system with a zero-g capability and based on the filter-capsule approach has been completed. This system is intended for monitoring the sterility of regenerated water in a spacecraft.

The principle of detection is based on measuring the increase in chemiluminescence produced by the action of bacterial porphyrins (i.e., catalase, cytochromes, etc.) on a luminol-hydrogen peroxide mixture. Since viable as well as nonviable organisms initiate this luminescence, viable organisms are detected by comparing the signal of an incubated water sample with an unincubated control. Higher signals for the former indicate the presence of viable organisms.

System features include disposable sealed sterile capsules, each containing a filter membrane, for processing discrete water samples and a tape transport for moving these capsules through a processing sequence which involves sample concentration, nutrient addition, incubation, a 4 Molar Urea wash and reaction with luminol-hydrogen peroxide in front of a photomultiplier tube. Liquids are introduced by means of a syringe needle which pierces a rubber septum contained in the wall of the capsule.

Detection thresholds obtained with this unit towards E. coli and S. marcescens assuming a 400 ml water sample are indicated below:

	Unincubated Cycle		Incubated Cycle	
	(Total viable + nonviable)		(Viable)	
	<u>Threshold</u>	<u>Processing Time</u>	<u>Threshold</u>	<u>Processing Time</u>
<u>E. coli</u>	60-100 cells/ml	36 min.	20-60 cells/ml	2.4 hrs.
<u>S. marcescens</u>	200 cells/ml	36 min.	150 cells/ml	2.4 hrs.

Suggested system modifications for approaching target sensitivities of <5 cells/ml (viable) and <10 cells/ml (total) are presented.

The zero-g capability of the Processing Station Assembly was demonstrated by operating the unit sideways and upside down.

A limited study of the shelf life of the required reagents indicated the following:

4M Urea - stable for at least seven weeks at ambient or 37°C .

Dextrose broth - stable for at least one month at ambient (in the dark).

Luminol and H_2O_2 (1%) Reagents - stable for at least one month at 37°C and six months at ambient.

It is to be expected that more extensive studies will reveal a useable shelf life for all these reagents of at least six months if stored in sealed containers in the absence of air and light. An indefinite shelf life of one year or longer might be achieved (for all except the H_2O_2) by storing the reagents as dry powders and reconstituting with water just prior to use. If properly prepared (in the absence of organics or metal impurities) the H_2O_2 reagent should be stable for at least one year at ambient temperature.

Section 2

INTRODUCTION

This comprises the final report of progress in development of a "Tape Cassette Bacteria Detection System," and is submitted in accordance with the requirements of Contract No. NAS 9-12548.

The objective of this contract was to develop an automatic continuous bacterial detection system for monitoring the sterility of re-generated water.

The principle utilized for detection involved measuring the increase in chemiluminescence produced by the catalytic action of bacterial porphyrins on a luminol-hydrogen peroxide mixture. Since viable as well as nonviable organisms initiate this chemiluminescence, viable organisms are detected by comparing the signal of an incubated water sample with an unincubated control. Higher signals for the former indicate the presence of viable organisms.

The device fabricated under the present program featured the filter-capsule tape transport concept. In this approach, sealed capsules, each containing a sterile membrane filter and dispensed automatically, are used for processing discrete water samples. Using protocols developed under an earlier contract *, sample and processing fluids are pressure-filtered through each capsule as it passes sequentially from one station to another. The liquids are introduced by means of a hypodermic needle which pierces a rubber septum contained in the wall of the capsule. The use of individual capsules rather than a continuous filter tape is preferred for sample processing in order to minimize cross-contamination between samples.

The tape transport system developed under the present contract and the results obtained with E. coli and S. marcescens are described in detail below.

* Developed for NASA/MSC under Contract No. NAS 9-11644, completed February 29, 1972. Processing sequence involves sample concentration (400 ml), nutrient addition, incubation, 4M Urea wash and reaction with luminol - H₂O₂ reagent.

Section 3

EXPERIMENTAL RESULTS

3.1 INSTRUMENTATION

3.1.1 Filter-Capsule

The major component and one which forms the basis for design of the instrument itself is the filter-capsule. An exploded view is shown in Figure 1.

Apart from the membrane filter, the capsule contains a window cap, capsule body and retainer sleeve, all molded out of Plexiglas (polymethyl methacrylate). A stainless steel support screen, silicone gasket and rubber septum comprise the rest of the capsule assembly.

In actual use, the needle used for introducing liquids into the capsule penetrates the rubber septum from the bottom. A groove in the body of the capsule connects the inlet with the reaction well.

In assembling the capsule (see Table 1), the window cap is first cemented to the body of the capsule. The membrane filter, support screen and gasket are then placed in the recess of the capsule body and compressed by the retainer sleeve, which is cemented in position. Finally, the septum is inserted in the capsule body. The assembled capsule is then outgassed at room temperature for one-half hour to remove any volatile components which might inhibit bacterial growth in the viable cycle. Prior to insertion in the capsule, the membrane filter is presoaked in 4 M Urea for 1/2 hour, rinsed in sterile distilled water and then air dried. This pretreatment removes most of the surface finishes and porphyrin-containing particulates from the filter.

Further pretreatment of the assembled capsule involves passing luminol - H_2O_2 reagent (Premix) through the capsule, washing with filtered distilled water and then air drying at pasteurization temperature. (See Laboratory Support Studies, Section 4 for details). Capsules prepared in this manner are suitable for use in the total and viable cycles. The capsule is then fastened to the 70 mm transport film by two retaining screws as shown in Figure 2. The film has been exposed and developed to a dense black to minimize the problem of "light piping", a property inherent in most plastics.

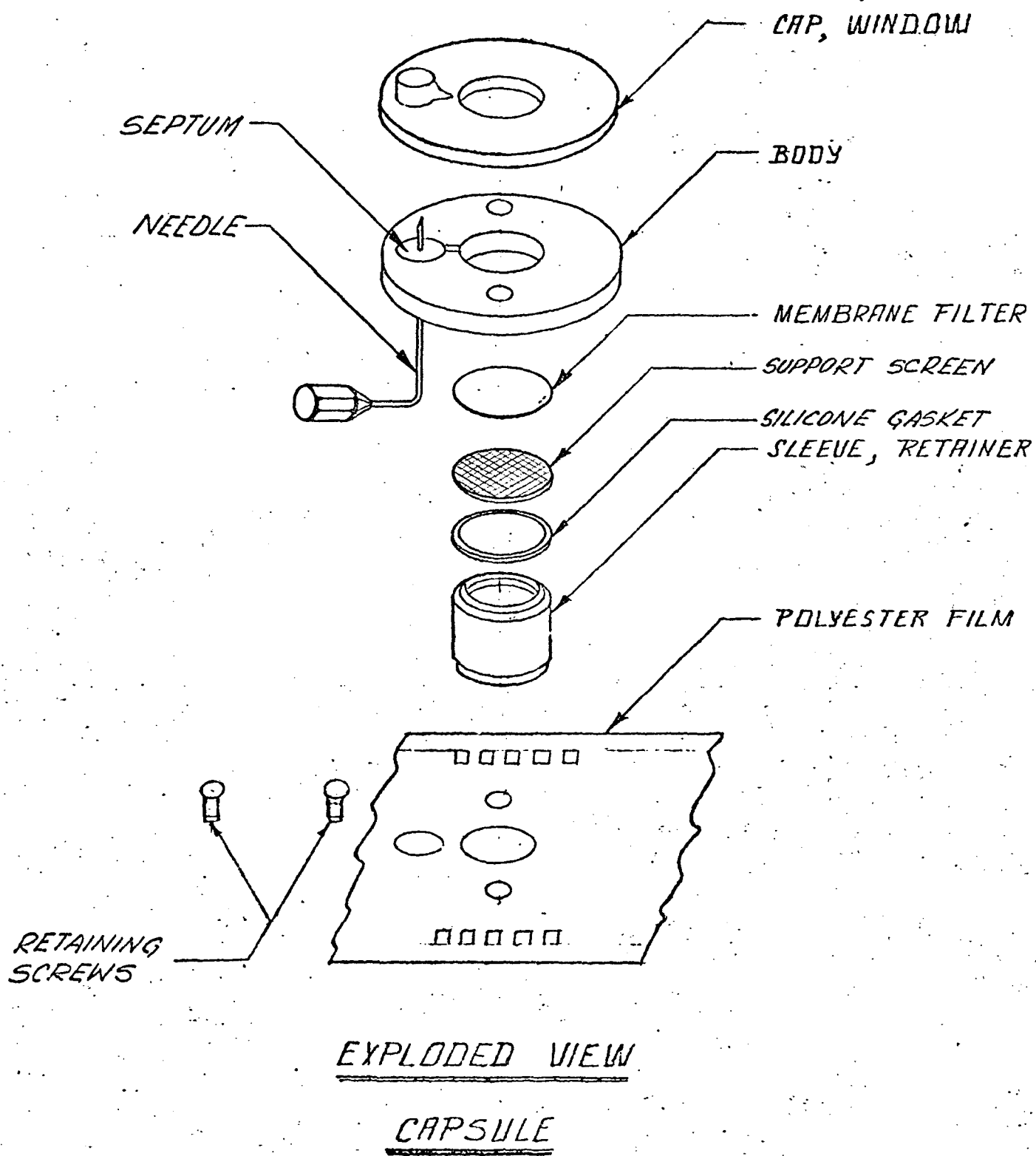
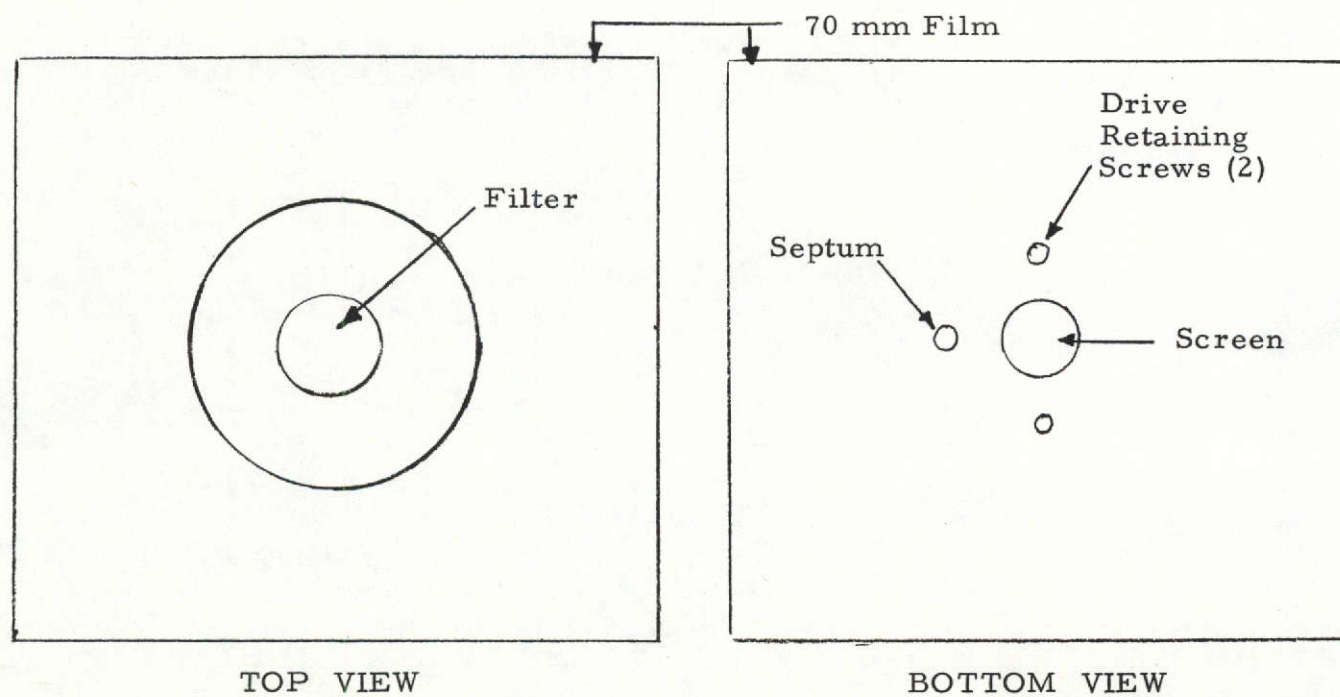
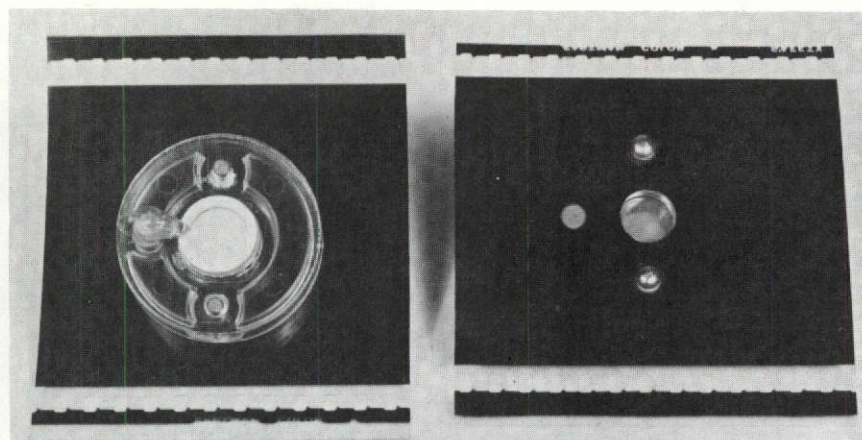


Figure 1

Table 1
CAPSULE ASSEMBLY
(Refer to Figure 1)

1. Holding window cap and capsule body (canal-side up) between thumb and forefinger, rotate capsule body until septum hole aligns with needle cavity (window cap).
2. Apply several drops of IPS (Industrial Polychemical Service, California) styrene adhesive (#2395) to perimeter of clamped parts. The glue fills the bonding area by capillary action. Maintain slight pressure around the bond for 1 minute.
3. The presence of air pockets can be detected by placing the assembly over a darkened area. Apply a drop or two of glue around the edge of the window area until the gaps are filled. Let capsule dry for 10 minutes.
4. Place the retainer sleeve on a cork (for convenience) with the bevel up. The undercut goes down around the cork.
5. Apply the more viscous adhesive, IPS styrene (#1807), with a spatula around the outside of the sleeve. Let it dry for 2 minutes.
6. Place the membrane filter in the window area, followed by the stainless steel screen and gasket.
7. Take the cork with the retainer sleeve and place into capsule cavity, (i. e., bevel end against gasket). Remove cork and push sleeve firmly for proper seal. Let dry for 10 minutes.
8. While secondary bond is drying, press septum material into position using prefabricated punch and die.
9. Remove excess septum material and any excess glue from retainer sleeve with a razor blade.
10. Place assembled capsule in vacuum desiccator (25" Hg) for one-half hour.



CAPSULE MOUNTED ON TRANSPORT FILM

Figure 2

A mechanical hand punch, used for stamping out the appropriate sized holes in the transport film at prescribed intervals is shown in Figure 3.

The laboratory support studies involved in developing this capsule are described in Section 4 (Laboratory Support Studies).

3.1.2 Integrated Detection System

The fully integrated bacteria detection system, shown in Figure 4, contains three major assemblies - an electronic control chassis, a pump and valve assembly and a processing station assembly. A brief description of each of these is given below:

a. Electronic Control Chassis (Figure 5)

This module is used for programming and signal processing. Individual components include two Agastat stepping switches (18 steps each) which control the sequential energization of the various load circuits, 1 and 3-hour timers (for sample concentration and incubation, respectively), a high voltage supply (for the PMT), a 24 volt power supply to drive the solenoids, a + 12 volt power supply for the electronics, relay and signal processing circuitry.

The system can be operated in a manual or automatic mode. The dials shown on the Control Panel in Figure 6, are individual potentiometers for varying the time interval of the individual steps in the processing sequence. The numbered toggle switches are for operating the system in a manual mode.

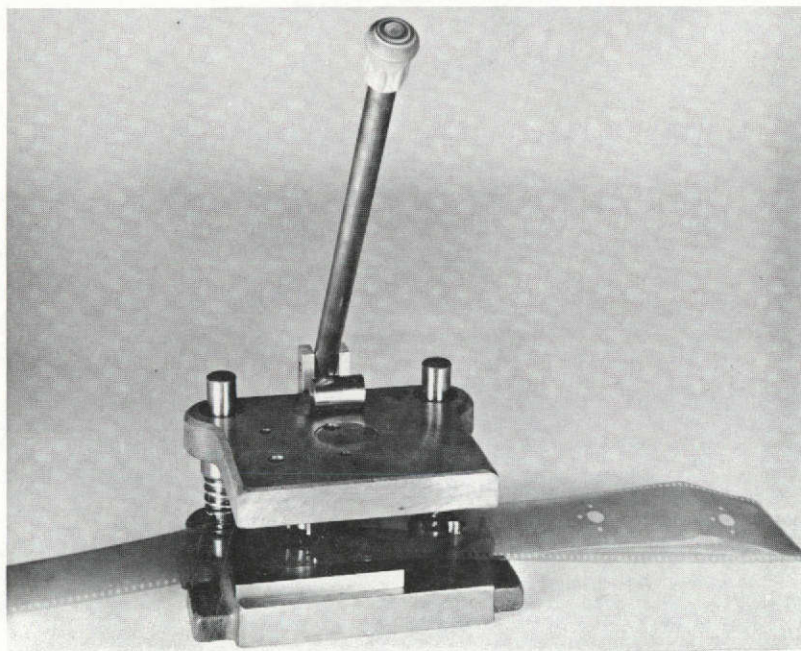
b. Pump and Valve Assembly (Figure 7)

This subsystem contains (7) peristaltic metering pumps, Chromatronix valves and solenoids for actuating the valves.

c. Processing Station Assembly (Figures 8 and 9)

This assembly contains the individual sample processing stations mounted on a single backplate (i. e., sample concentration, nutrient addition, incubator, Urea wash, reaction and readout). Except for the incubator, each station has an inlet line which leads to the syringe needle and an outlet line which exhausts to the waste pump.

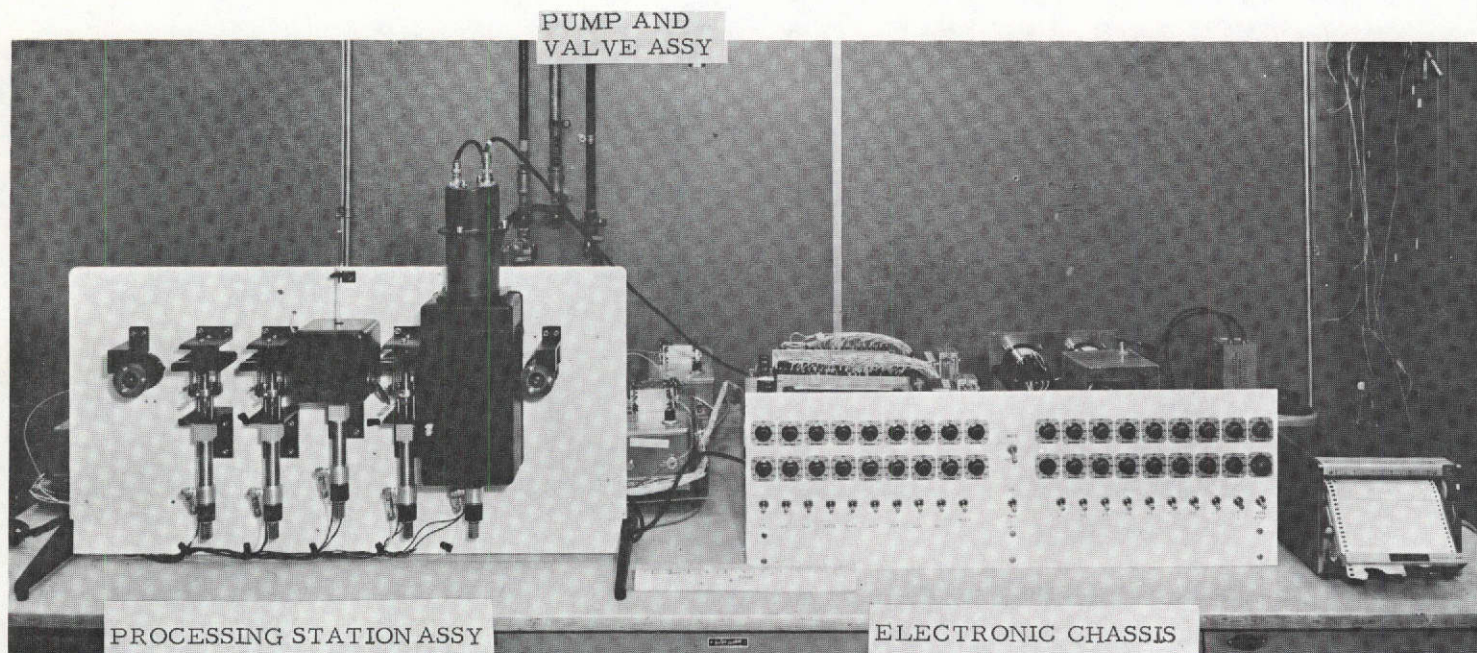
Indexing of the capsule from one station to the next is accomplished by means of a sprocket arrangement driven by an adjustable rack and pinion (mounted on back panel, Figure 10).



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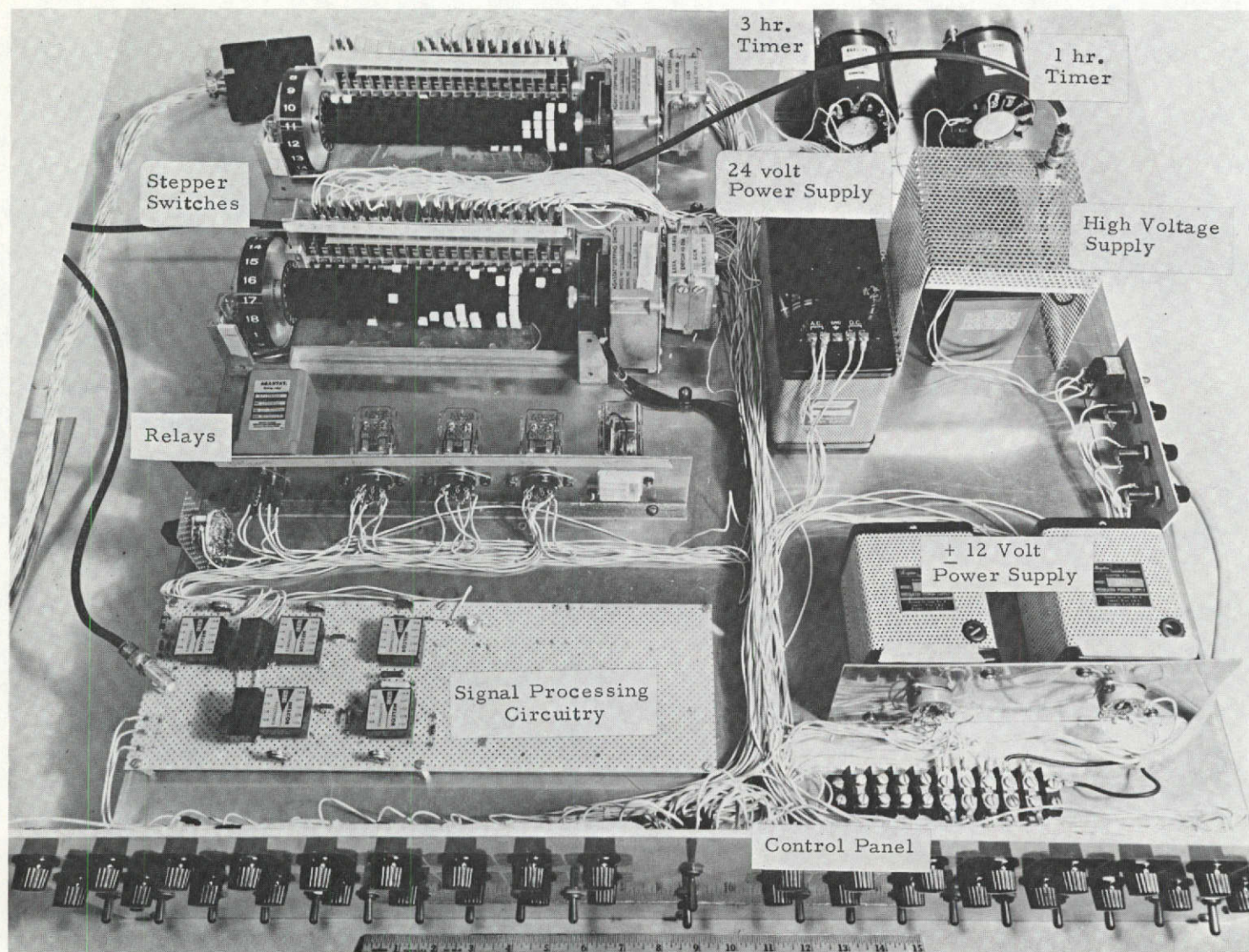
MECHANICAL HAND PUNCH

Figure 3



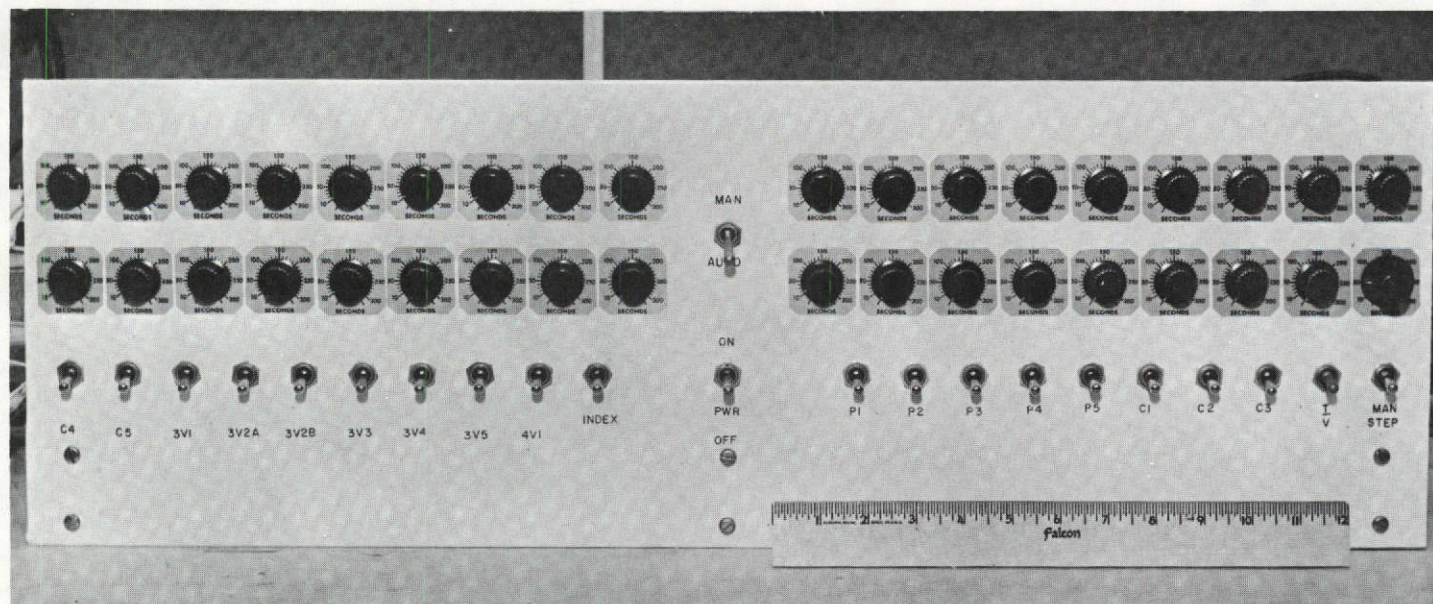
TAPE TRANSPORT BACTERIA
DETECTION SYSTEM

Figure 4



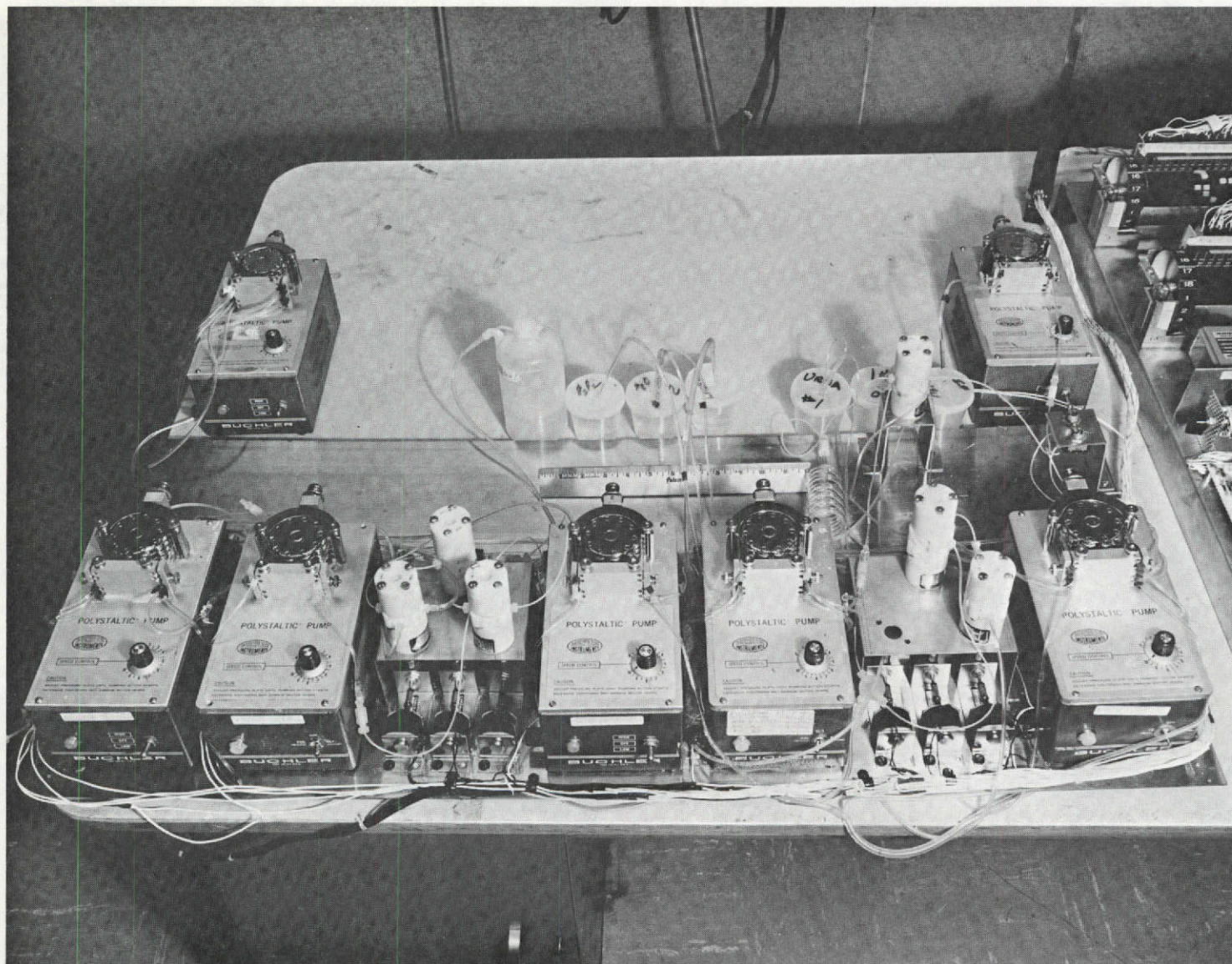
ELECTRONIC CONTROL CHASSIS

Figure 5



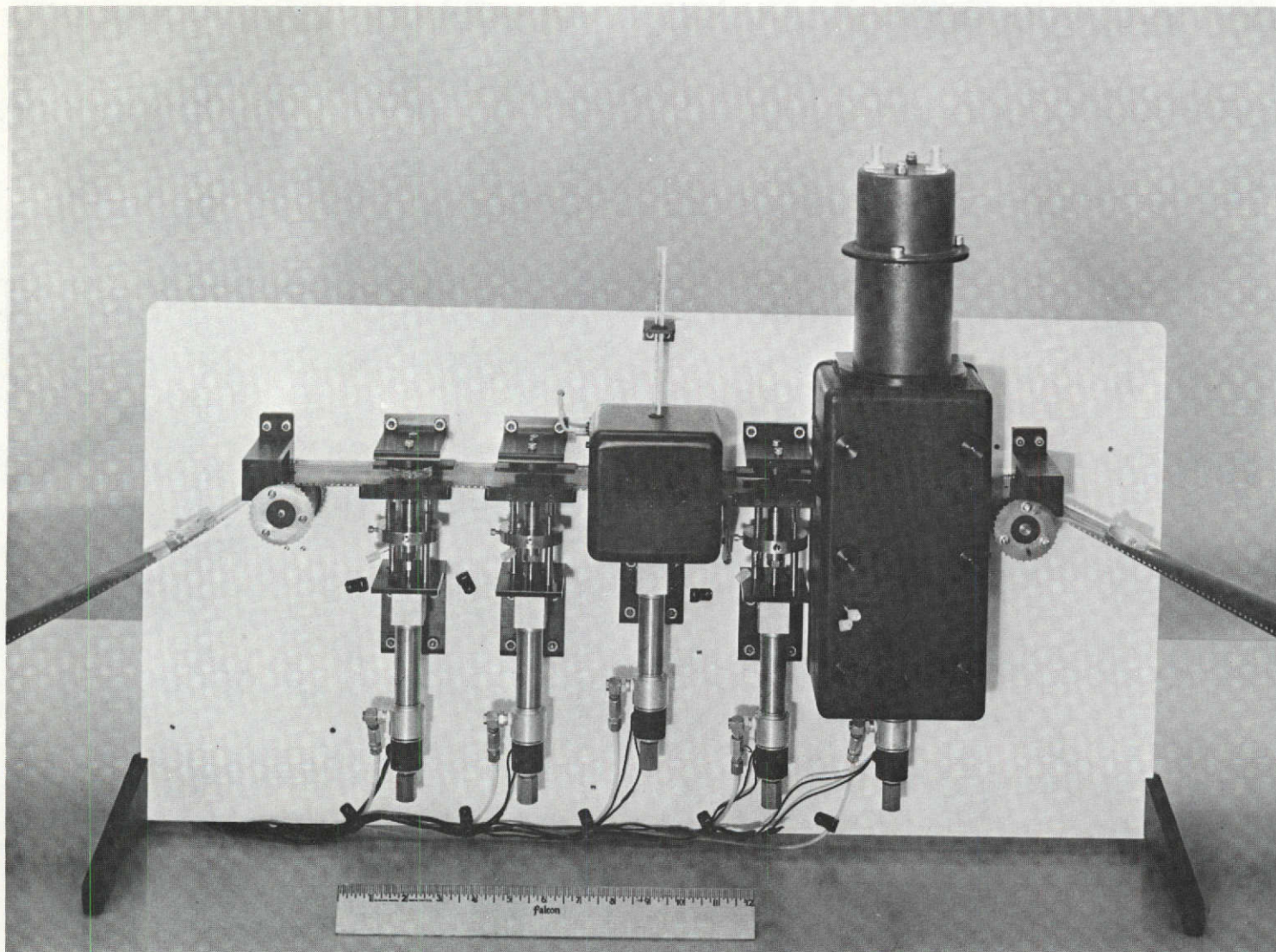
CONTROL PANEL

Figure 6



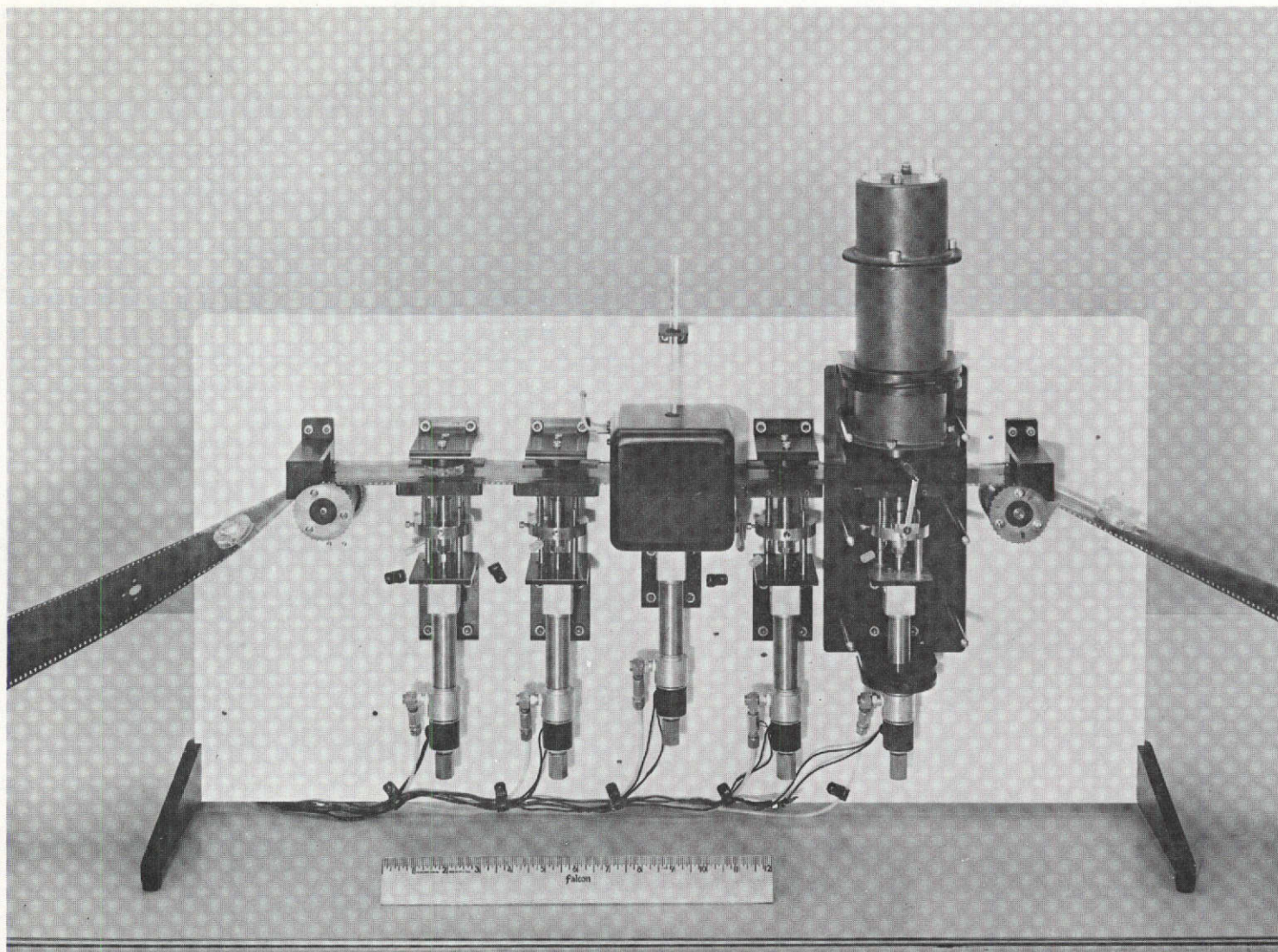
PUMP AND VALVE ASSEMBLY

Figure 7



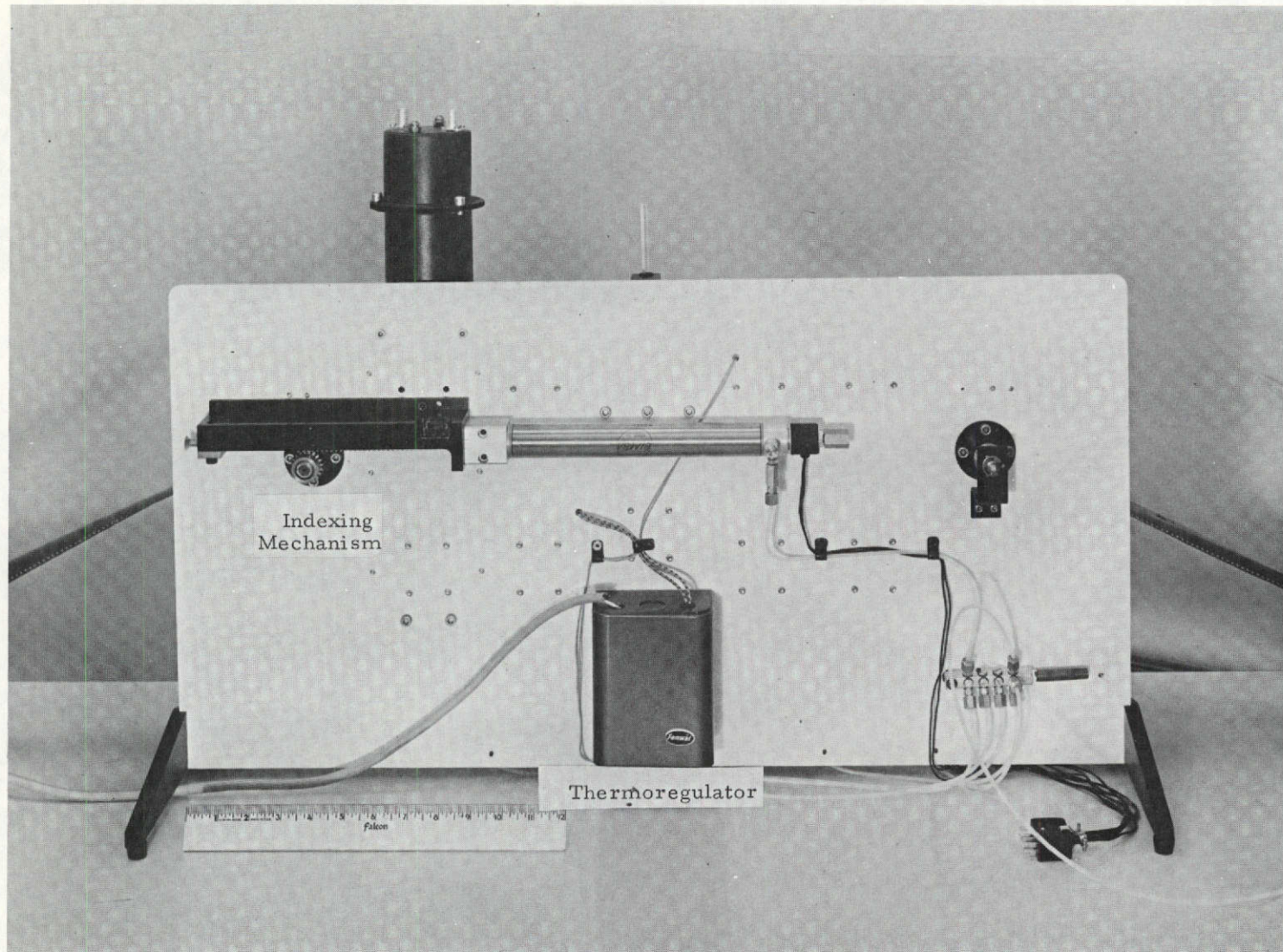
PROCESSING STATION ASSEMBLY

Figure 8



PROCESSING STATION ASSEMBLY
(LIGHT SHIELD REMOVED)

Figure 9



PROCESSING STATION
ASSEMBLY
(BACK PANEL)

Figure 10

The principle of operation of an individual processing station can be illustrated by reference to the schematic shown in Figure 11. The operational sequence which occurs when the station is activated is summarized in Table 2. Briefly, on the upward stroke of the piston rod the capsule is centered when it is pushed up against the capsule cover (latter contains a recessed rim). The needle then penetrates the rubber septum as the bottom of the capsule is engaged by the "O" ring on the piston rod (thereby, affecting a seal). When the piston rod is lowered, the reverse sequence occurs.

A schematic of the readout station (Station 5) is shown in Figure 12. An automatically controlled butterfly shutter mounted inside a reflecting metal cylinder serves to protect the photomultiplier tube (PMT) from stray light that may be introduced when the capsule passes into the light housing assembly.

3.1.3 Processing Sequence (Refer to Figure 13)

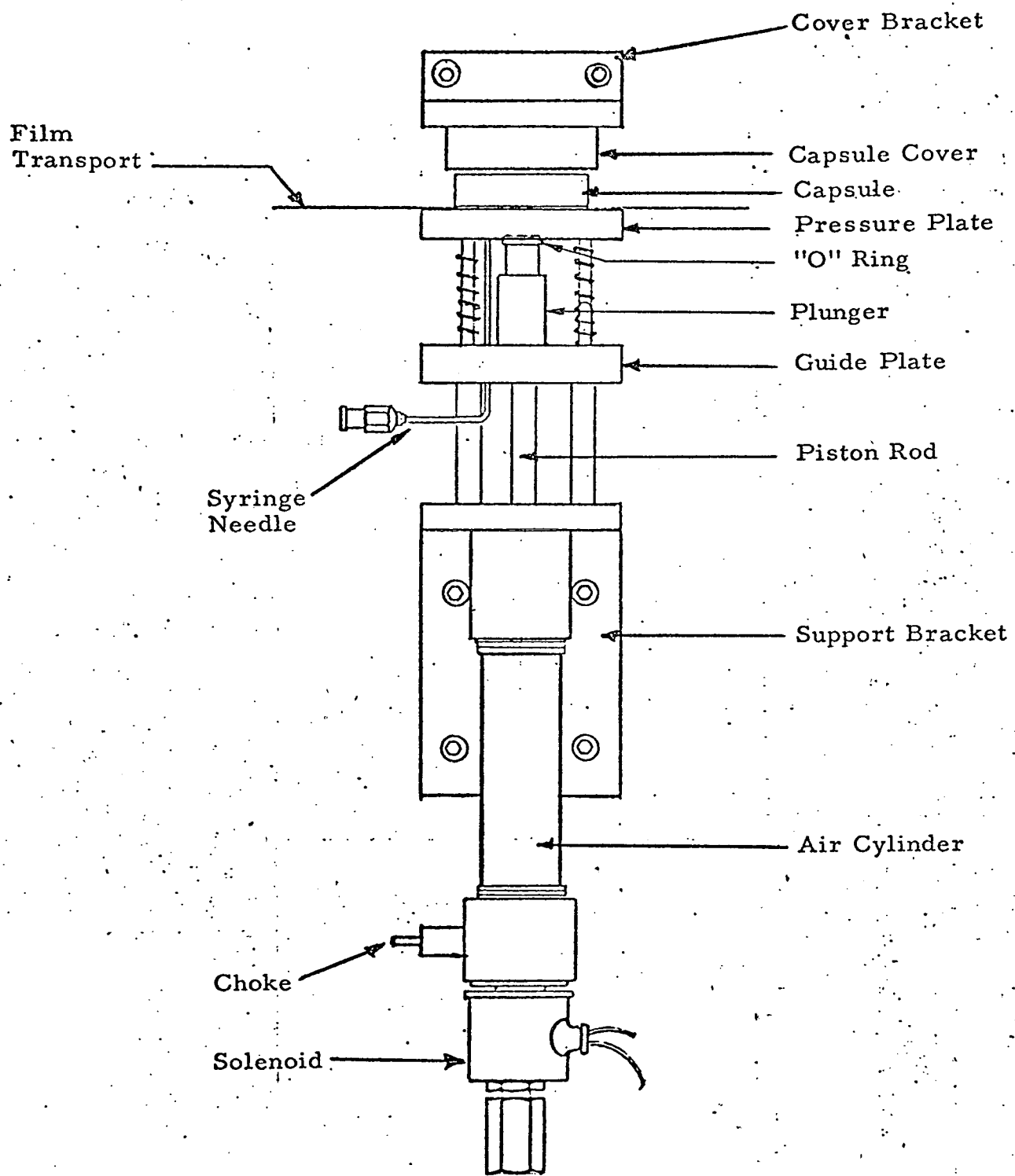
The following functions are performed at each of the stations (detailed operational procedures for the instrument are given in Appendices A through C).

Station 1. A 400 ml water sample is pressure-filtered through the capsule (liquid enters via syringe needle and effluent from capsule passes out through a port in front of guide plate).

Station 2. A small amount of dextrose nutrient is pressure-fed through the syringe needle into the capsule. As this capsule passes to Station 3, another (empty) capsule directly behind it moves into position at Station 2. A Urea solution is passed through the syringe needle followed by a sterile water rinse. This Urea water rinse is intended to sterilize the syringe needle between samples and prevent cross-contamination (see Section 4 for details).

Station 3. Capsule containing deposited organisms is incubated for two hours at 37°C (bottom of capsule sealed off during the incubation to prevent drying out of nutrient).

Station 4. 4M Urea solution is pressure-fed into capsule (via syringe needle) to wash out nutrient.



PROCESSING STATION
(SCHEMATIC)

Figure 11

Table 2

Operational Sequence for Processing Station

1. Programmer energizes solenoid.
2. Air enters cylinder through choke.
3. Piston and rod extends pushing guide plate up.
4. Guide plate and pressure plate move upward moving capsule up against capsule cover.
5. Guide plate continues upward movement against spring tension around pressure plate guides.
6. Guide plate continues movement upward to end of piston stroke at which time the syringe needle will have penetrated septum and the "O" ring on the plunger will have entered the recess of the capsule and affected a seal.
7. Turn exhaust pump on.
8. Turn feed pump on (to pressure-filter sample via the syringe needle).
9. After prescribed amount of liquid has passed through the capsule, turn feed pump off.
10. Programmer de-energizes solenoid and air in cylinder is vented.
11. Guide plate retracts withdrawing needle and plug (any residual liquid trapped beneath the filter screen in the capsule and the sealing plug will be withdrawn to waste by the exhaust pump which is still on).
12. Turn exhaust pump off.
13. Index capsule to the next station.

PMT
Housing

PMT

Back Plate

Shutter
(Open)

Bell
Crank

Reflecting
Metal
Cylinder

Capsule

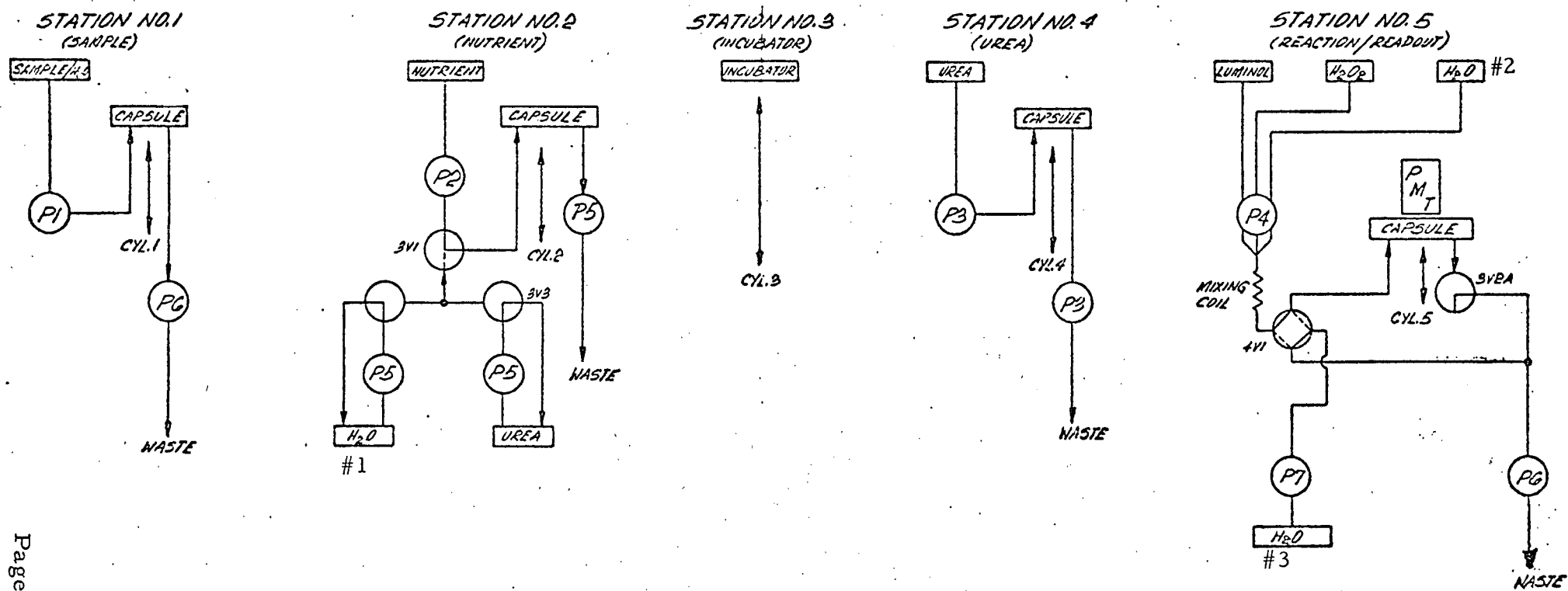
Film

Bell
Crank
Rod

Shutter
Actuator

PMT HOUSING ASSEMBLY

Figure 12



Station 5. Luminol-hydrogen peroxide reagent mixture is introduced into the capsule via the syringe needle and reacted with the deposited organisms on the filter. The generated light is monitored by a photomultiplier and the processed signal appears on a strip chart recorder.

Following reaction with Premix reagent, sterile distilled water is passed through the capsule (via the syringe needle) to remove residual reagent which might react prematurely with the next sample capsule.

The time interval involved for each of the processing steps in a total and viable cycle is summarized in Table 3. It is evident that approximately 1/2 hour is required to complete a cycle for total bacterial count and approximately an additional 2-1/2 hours for the viable cycle.

The physical dimensions and operating parameters of the integrated bacteria detection system are summarized in Table 4. Shown for comparison are the reagent requirements for the Water Monitor developed by AMB for the Langley Research Center, Contract NAS 1-10382. In the latter system, the bacteria are concentrated on a rotating reusable filter, washed and then backwashed into a glass tube where reaction with Premix occurs in front of a PMT. The reagent requirements for this mode of operation are seen to be considerably greater than for the tape transport system.

The shelf-life of the various reagent components have also been characterized, (see Section 4) and shown to be stable for at least 1 to 6 months.

3.2 INSTRUMENT RELIABILITY

In evaluating the integrated system, a number of mechanical deficiencies were noted which affected its operational reliability. Some of these problems have been largely resolved. Their current status is summarized below:

a. Capsule Plugging - due principally to blockage of the filter pores and inlet channel by excessive glue wicking to these areas. This problem has been largely resolved by using a more viscous glue (at the retainer sleeve) and a modified capsule assembly procedure (see Table 1).

Table 3
Processing Sequence

<u>Station</u>	<u>Function</u>	<u>FLOW RATE</u> <u>ml/min.</u>	<u>DURATION, MIN.</u>	
			<u>TOTAL</u>	<u>VIABLE</u>
1	Sample Concentration (400 ml)	20	20	20
2	Nutrient Added	5.4	1	1
	Urea Cleanup (Second Capsule)	3.8	3	3
	Water Cleanup (Second Capsule)	3.8	3	3
3	Incubation	---	0	120
4	Urea Wash	6.0	3	3
5	Premix Reaction	6.1	5*	5*
	Water Wash	3.7	0.5	0.5
TOTAL TIME			36 min.	156 min.
(~2-1/2 hrs.)				

* Premix reagent on for only 10 sec., flow then turn off and reaction permitted to proceed for remainder of 5 min.

AMB Water Monitor Specifications

* Reagent requirements for Langley Water Monitor (Contract NAS 1-10382)
 ** Reagent preparation is described in Appendix D.

b. Capsule Leakage - arose from a number of sources; namely, 1) channeling in glue-line, 2) failure at the needle - septum and septum-capsule interfaces, 3) inadequate seating of the "O" rings on some of the piston rods, or 4) an insufficient exhaust vacuum on the waste manifold. This problem has been largely reduced (from ~30% initially to < 5% currently) by use of 1) a more effective gluing procedure, 2) finer syringe needles (i. e., a 25-guage needle reinforced by a 20-guage metal sleeve), which reduce the probability of leakage on repeated punctures, 3) sleeve guides which ensure centering of the needle on penetration, 4) a stiffer septum material, 5) an increased vacuum on each of the waste manifolds. The use of larger "O" rings on the piston rods at each station should also provide a better seal and minimize leakage at the exit side of the capsule.

c. Needle Plugging - resulting from small slivers of rubber sheared from the septum, is still a major problem. The frequency of plugging was found to be related not only to the type of septum material employed but also to 1) the size and pitch of the needle point, 2) the speed and angle of penetration and 3) the liquid flow rate. Reduced plugging occurs with use of 1) softer, less friable septum materials, 2) finer needles (i. e., 25-guage vs. 20-guage) with a larger pitch of the point (angle formed between vertical wall and cutting ellipse at needle point should be greater than 22°), 3) centered and more rapid penetration of the septum and 4) higher flow rates. The use of a solid pointed needle with the hole at the side should help resolve this problem.

d. Indexing Problems - repeated indexing does not always advance capsule the required distance. When this happens, plunger on air cylinder does not come up all the way. Possible sources of this misalignment are 1) station components drift out of alignment or 2) misalignment of the window cap with respect to the capsule body on assembly. The use of indexing pins between these two capsule components should eliminate the problem from this source.

e. Light Leak at the Readout Station - significant light leakage (~ 25 volts) was observed when the capsule was in position (for reaction) and the shutter opened. Better than 90% of the leakage was occurring at the exit slit of the light housing and the balance from the entrance slit (see Figure 14). Draping a black cloth over both of these areas eliminated the leakage indicating that "light piping" of the transport film was negligible. Additional shielding will be required to reduce the leakage to an acceptable level (i. e., < 1 volt).

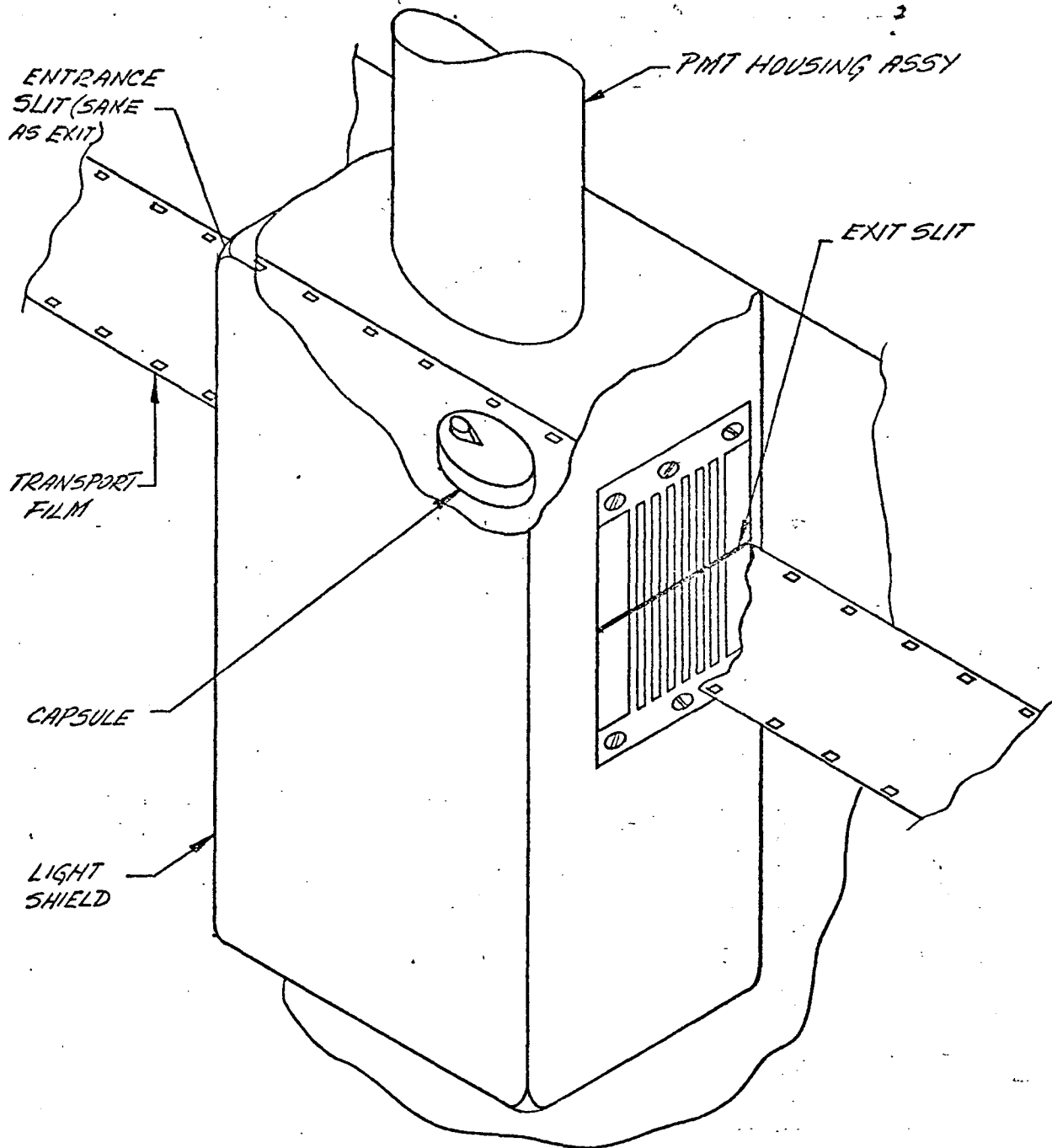
3.3 UNINCUBATED CYCLE

In the course of the program three different methods were evaluated for reacting the organisms with Premix reagent at the readout station. These included (a) Continuous Flow: a method in which the reagent is flowed through the filter under a partial vacuum for five minutes (see Figure 15), (b) (Wet) Stopped-Flow: a technique in which the reagent was flowed through the filter for only a few seconds (long enough to displace the residual 4M Urea in the capsule cavity with Premix*) and then turned off. The reaction was then permitted to proceed for the remainder of five minutes (see Figure 15) and (c) (Dry) Stopped-Flow: a method in which the residual liquid Urea in the capsule was removed by blowing air through the capsule prior to reaction with Premix using the "stopped-flow" technique.

3.3.1 Reagent Flow Through Filter

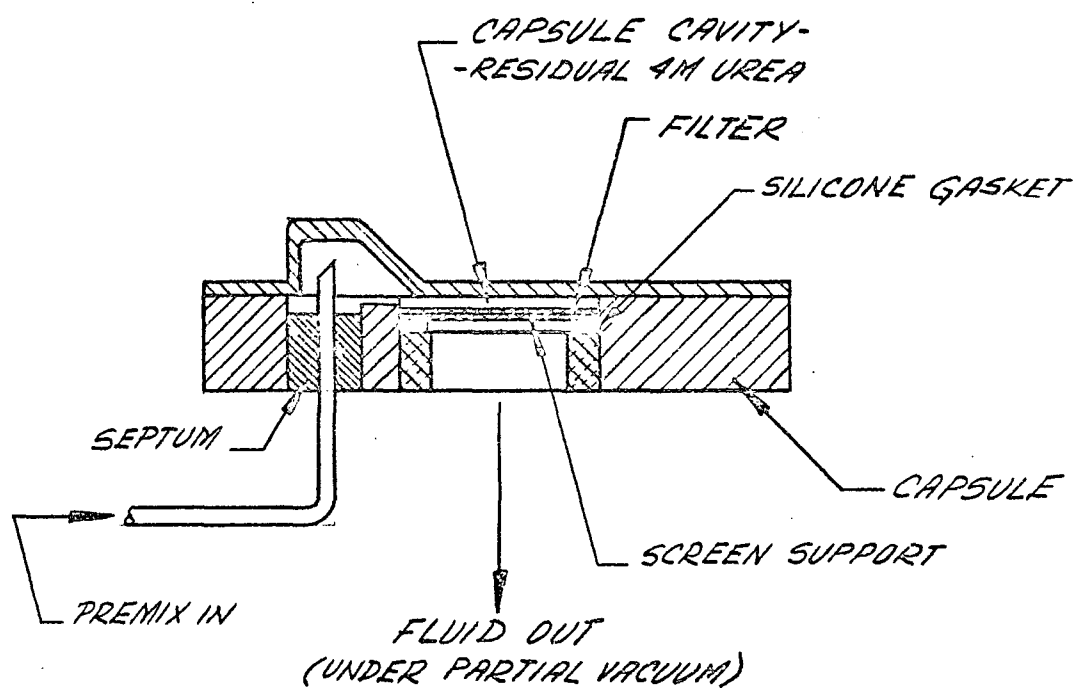
The first method produced results that were more erratic and less sensitive than the other two. The large variability in the data can be attributed to nonuniform flow of reagent through the filter. The signal amplitude is highly dependent on flow rate and variations in the latter could be produced by bubble buildup on the filter (which would affect the pressure drop and hence flow rate through the filter), variation in the vacuum source on the exhaust line, variations in filter porosity and partial plugging of the needle. The accumulation of bubbles on the filter could decrease the amount of light reaching the PMT by diffracting the generated light to the sides of the capsule. Since there is no light gathering skirt around the perimeter of the capsule, this portion of the light would be lost.

* After the 4M Urea wash at Station 4, some liquid Urea solution remains in the capsule cavity.



PMT HOUSING ASSY & LIGHT SHIELD.

Figure 14



REAGENT FLOW IN CAPSULE

Figure 15

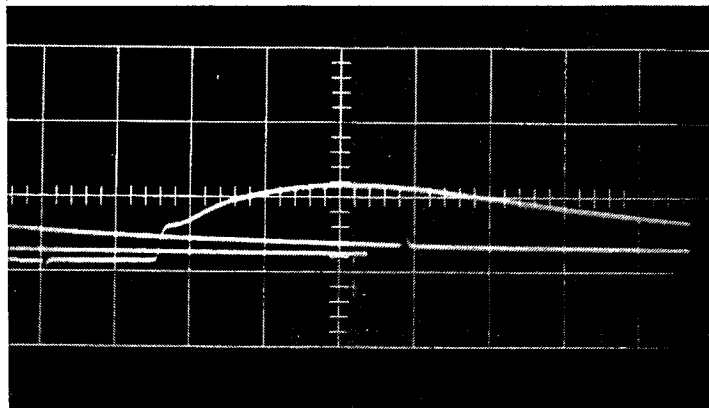
The lower sensitivity observed with this method could be due to leaching of porphyrins from the bacteria by the Premix as it passes through the filter. Studies in AMB laboratories indicate that the leaching is almost instantaneous and would be expected to be more severe the higher the flow rate.

Both of these shortcomings (i. e., variable flow and loss of porphyrins by leaching) might be eliminated by flowing the reagent across the surface of the filter. To offset the loss in signal due to leaching, at least an 8-second residence time within the reaction chamber would be required. The reason for the latter requirement becomes evident by reference to the oscilloscope traces shown in Figure 16. These were obtained at AMB laboratories by injecting Premix reagent into a test tube containing a bacterial suspension of these organisms in front of a PMT. The time - intensity profiles obtained with E. coli and S. marcescens indicate that approximately 5 and 8 seconds, respectively are required for these organisms to reach maximum luminescence intensity. The residence time for the Premix reagent in the previous program (NAS 9-11644) was approximately 7 seconds.

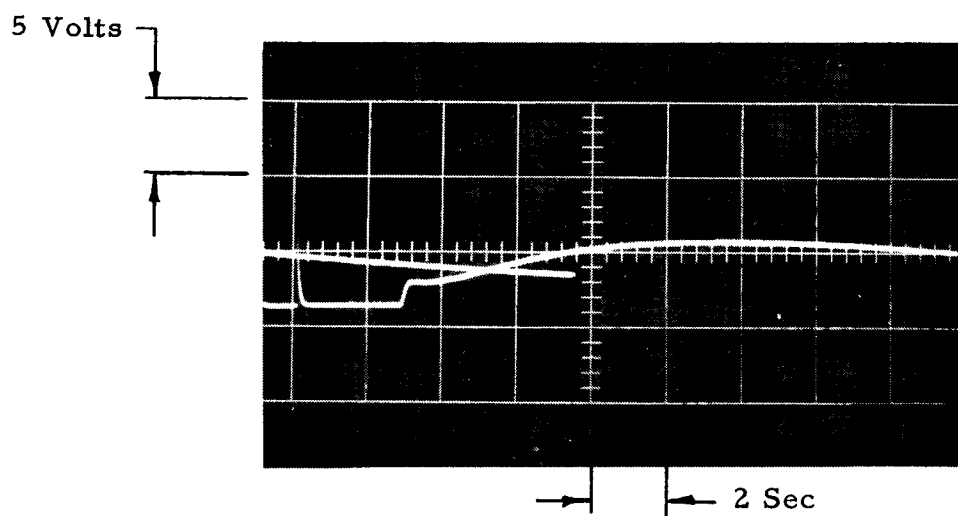
While reagent flow across the tape would be more advantageous than flow through the tape, this would entail some major changes in the present system and capsule design. It was thought, however, that "flow across tape" might be approximated by the "stopped-flow" technique described earlier. The results obtained using this approach are described below.

3.3.2 "Stopped Flow" Technique (Wet)

Typical traces obtained for E. coli and S. marcescens using this technique are shown in Figures 17 and 18. The chart speed has been stepped up to obtain an expanded time scale. The instrument was operated in a manual mode and the Premix flow turned off when the signal reached 5 volts. It is evident from the traces for E. coli and S. marcescens that the luminescence intensities continue to increase and reach a maximum 5 and 8 seconds, respectively, after addition of the Premix to the capsule. In both instances, the luminescence decays very slowly.



E. coli - 4.1×10^4 cells/ml

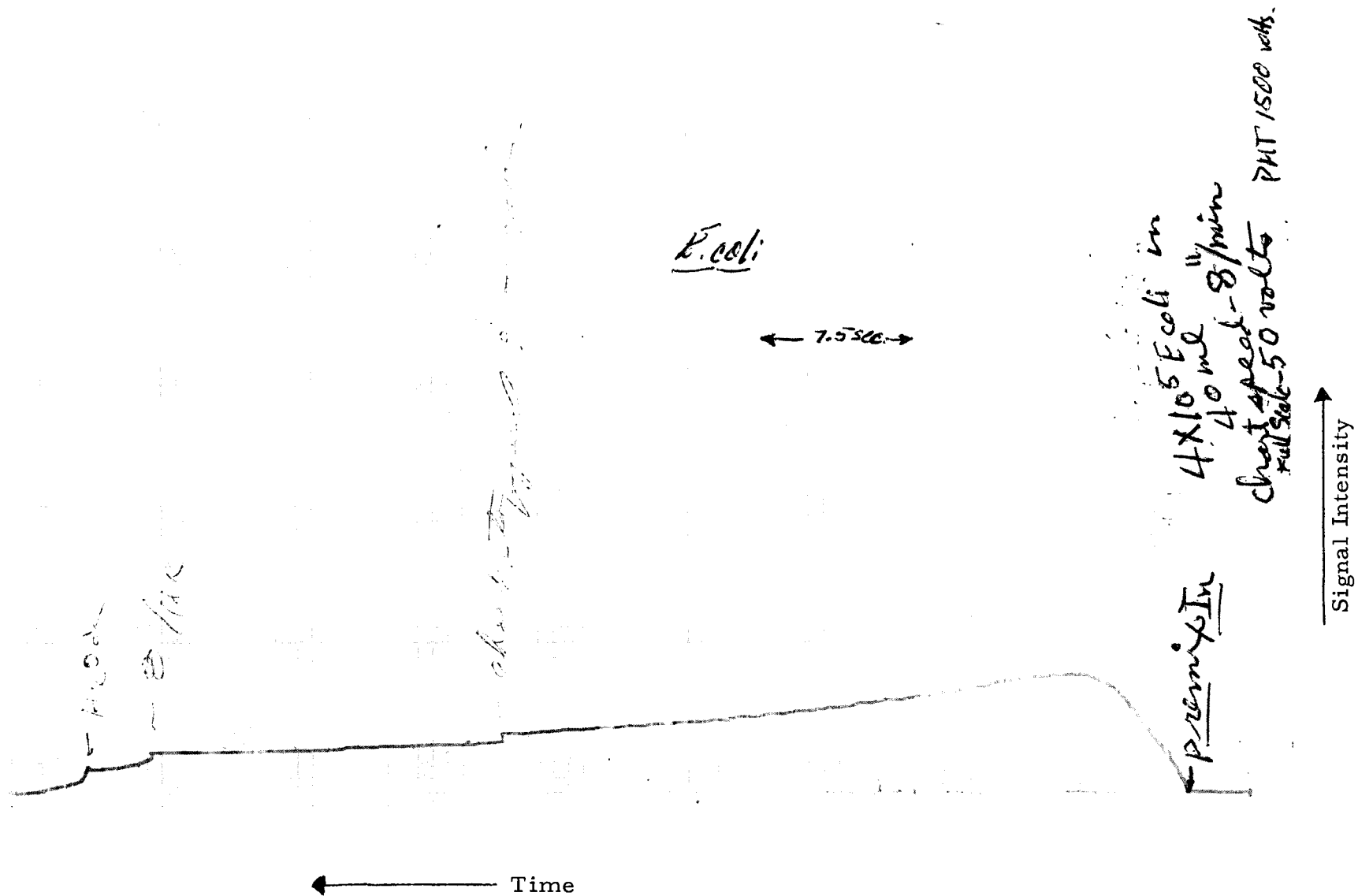


S. marcescens - 2.6×10^5 cells/ml

OSCILLOSCOPE TRACES

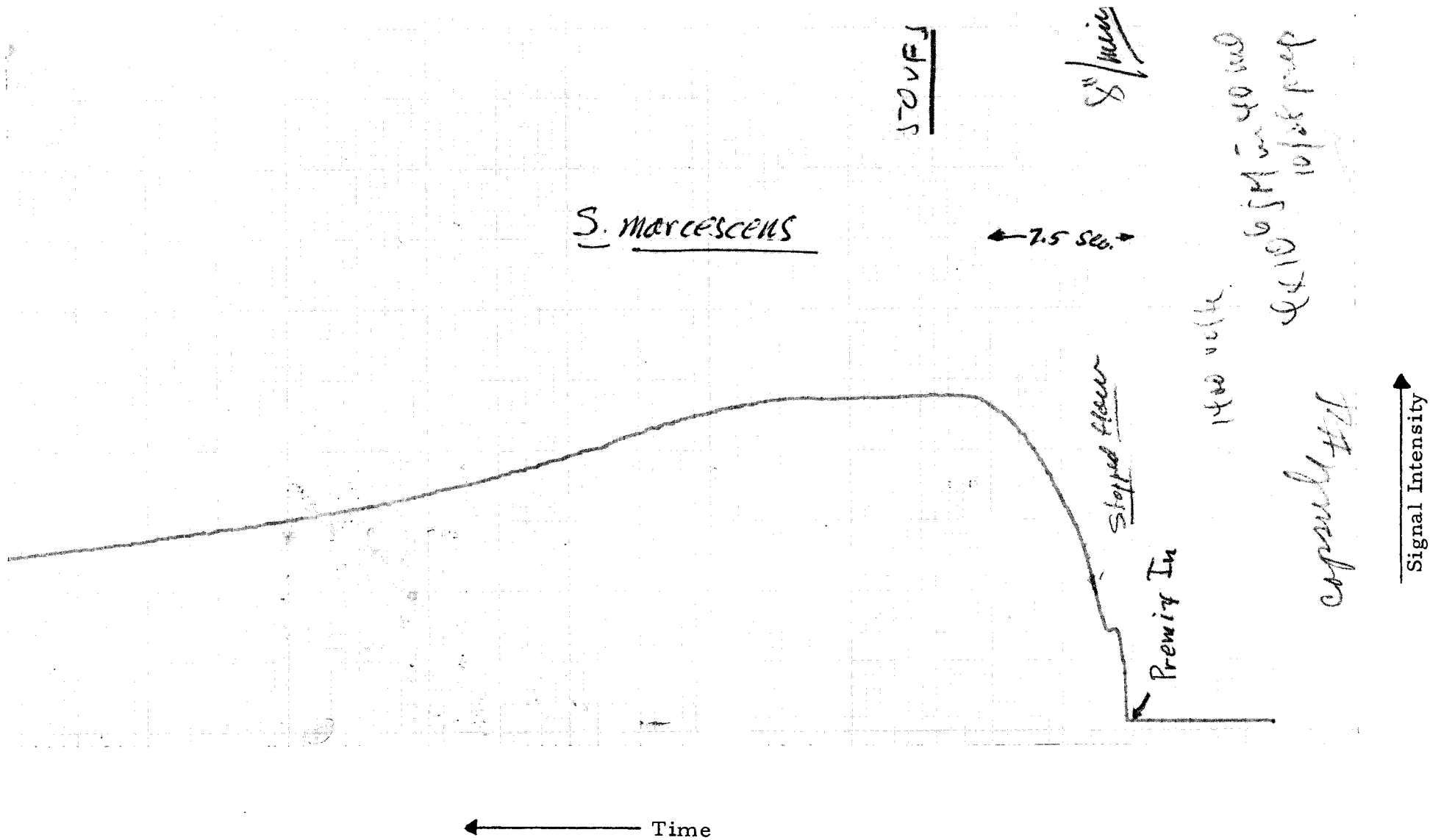
(Abscissa - 2 sec/cm; Ordinate - 5 volts/cm)

Figure 16



SIGNAL INTENSITY VS. TIME - E. coli

Figure 17



SIGNAL INTENSITY VS. TIME - S. marcescens

Figure 18

The signal response obtained for E. coli and S. marcescens in the unincubated cycle with the stopped-flow technique (in both manual and automatic mode) are summarized in Table 5. Data obtained in the previous program (with reagent flow across the tape) are shown for comparison. Using the data shown in the next to the last column as a basis for comparison, on the average, the sensitivity obtained with the (wet) stopped-flow technique is only about one-half as good as that obtained by flowing reagent across the tape. The apparent lower sensitivity with the stopped-flow technique is probably due principally to loss of porphyrins by leaching, as mentioned earlier. While leaching also occurs in the method employing reagent flow across the tape, the volume of the reaction cell and flow rates were such that the residence time of bacteria and reagents in the reaction cell (and within view of the PMT) was approximately 7 seconds, which is optimum for capturing the maximum intensity of the E. coli.

Partial blocking of the filter surface by entrapped bubbles, which has a greater probability of occurring with the stopped-flow technique, could also prevent complete reaction within the first 10 seconds, the critical reaction period.

Table 6 shows some data obtained by a stopped-flow technique in which the capsule was vented of any entrapped liquid Urea by blowing air through the capsule (with hand syringe) prior to reaction with Premix reagent. In addition, the small section of line connecting the shut-off valve (from the reagent reservoir) and the syringe needle were also (hand) filled with fresh Premix reagent prior to initiating reaction). This set of conditions approximates those utilized in the previous tape transport program where no liquid Urea remained on the tape prior to reaction with Premix at last station. The results in Table 6, indicate that the net signals obtained with this "dry stopped-flow" technique are identical to those obtained in the previous program. Apparently, entrapped liquid in the capsule could lead to channeling of reagents and incomplete reaction. This might be avoided by incorporating a step in the processing sequence in which filtered air automatically purges the residual Urea from the capsule after the Urea wash at the fourth station.

Table 5

SIGNAL RESPONSE - UNINCUBATED CYCLE

<u>Total Challenge</u>	<u>Mode</u> ***	<u>Sample#</u> <u>Signal V</u>	<u>Water</u> <u>Signal V</u>	<u>Net</u> <u>Signal V</u>	<u>Cells/</u> <u>ml/Volt</u> *	<u>Ref.</u> <u>Data</u> <u>Sheet</u> ##
A. <u>Stopped-Flow (Wet)</u>						
8×10^5 <u>E. coli</u>	Manual	21	4	17	118	66
8×10^5 <u>E. coli</u>	Auto	25	4	21	95	66
8×10^5 <u>E. coli</u>	Auto (Upside Down)	19	4	15	133	66
8×10^5 <u>E. coli</u>	Auto (On Side)	29	3	26	77	68
				av = 20 (S=+ 5)	av 106	
4×10^5 <u>E. coli</u>	Manual	14**	8**	6**	167	53
				(S=+ 1.4)		57, 62
4×10^5 <u>S. marcescens</u>	Manual	11**	6**	5**	200	60, 62
				(S=+2.6)		
B. <u>Flow Across Tape</u>^{oo}						
4×10^5 <u>E. Coli</u>	Manual	27	10	17	60	
				(S=+ 1)		

* Assuming total bacterial challenge contained in a 400 ml sample volume.

Signal maximum occurring at least 5 seconds after contact with Premix.

** Average four runs for E. coli, three runs for S. marcescens.

*** PMT 1500 volts for stopped flow, 1450 V for flow across tape.

oo Previous Contract NAS 9-11644.

Data sheets are contained in a separate volume entitled, "Data Sheets for Tape Transport Bacteria Detection System" Contract NAS 9-12548, dated 27 April 1973

TABLE 6

SIGNAL RESPONSE - UNINCUBATED CYCLE (STOPPED-FLOW - DRY*)

<u>E. coli</u> <u>Total Challenge</u>	<u>Mode</u>	<u>Sample</u> <u>Signal, V</u>	<u>Water</u> <u>Signal, V</u>	<u>Net</u> <u>Signal, V</u>	<u>Cells/ml/volt</u> **	<u>Ref.</u> <u>Data</u> <u>Sheet</u>
5 x 10 ⁵	Manual - Upside ‡ down (Hand Inject)	23	3	20	62	65
5 x 10 ⁵	Manual	23	3	20	62	64
5 x 10 ⁵	Manual	25	4	21	60	64

* Residual Urea removed by blowing air through the capsule with a hand syringe.

** Assuming total bacterial challenge contained in a 400 ml sample volume.

‡ Bacteria introduced into capsule with hand syringe and then brought to Station 5 for readout; PMT at 1500 V.

3.4 Incubated Cycle

Data obtained with the "stopped-flow" technique for E. coli and S. marcescens in the viable cycle are summarized in Table 7. The data indicate that with the stopped-flow technique, ~21-62 cells/ml of E. coli are required to produce a net signal of one volt. With S. marcescens, the number is considerably greater.

3.5 Operation in an Automatic Mode

The instrument has been successfully operated in an automatic mode using the program sequence shown in Tables 8 and 9. The data are comparable to those obtained with the manual mode of operation (see Tables 5 and 7). Problems of needle plugging and failure of the piston rod to come all the way up and engage the capsule (due to component misalignment) were occasionally observed. One major difference between the two modes of operation occurs at the last station. Whereas, in the manual mode the Premix flow is turned off as soon as the recorder pen reaches 5 volts, in the automatic mode the flow duration is 10 seconds. While this normally is adequate, temporary plugging of the needle (with blockage being released as the pressure builds up) can introduce some variability in the flow duration. The incorporation of a voltage level detector and relay into the circuitry could be used to stop the reagent flow automatically when the signal voltage reached 5 volts.

Referring to Table 8, two time periods are indicated for Step Nos. 13 and 14. Both have been used. The decreased Urea cleanup and increased water wash at Station 2 were used during the demonstration for the viable run when some questions arose as to whether all of the Urea was being removed. Additional studies are needed to optimize the post-wash cycle at Station 2.

Table 7

SIGNAL RESPONSE - INCUBATED CYCLE

<u>Bacterial Challenge</u>	<u>Mode</u>	<u>Unincubated Sample, V.</u>	<u>Incubated Sample, V.</u>	<u>Net Signal, V.</u>	<u>Viable Cells/ml/Volt**</u>	<u>Ref. Data Sheet</u>
A. <u>Stopped Flow (Wet)</u>						
2 x 10 ⁵ <u>E. coli</u> (80% Via.)	* Manual	4	23	19	21	56
	(PMT 1350V)					
2 x 10 ⁵ <u>E. coli</u> (80% Via.)	* Automatic	6	12.5	6.5	62	68
(Without hemin)	(PMT-1450V)					
4 x 10 ⁵ <u>S. marcescens</u>	Manual	4	8	4	150	63
(60% Via.)	(PMT-1400V)					
(Without hemin)						

* The percent viable of test sample was determined by actual plate count (standard pour plate method) at time of measurement.

** Assuming total viable bacterial challenge contained in a 400 ml sample volume.

18 STEP
SINGLE TIME-
BASE CHARTSTEPPER #1
11-21-72

STEP NUMBER	CONTROL TO ADVANCE	DWELL TIME IN SECONDS	DESCRIPTION OF OPERATION	<div> PUMP #1 PUMP #2 PUMP #3 PUMP #4 PUMP #5 CYLINDER #1 CYLINDER #2 CYLINDER #3 VALVE 3V1 VALVE 3V3 VALVE 3V4 PUMP #6 INDEX 20-MIN. TIMER 2-HR. TIMER SWITCH TIMER Energize Pin 8 Timer 2 Energize Pin 8 Timer 1 Switch Pin 7 Timer 1&2 </div>																				NO OR NC CONTACT	CIRCUIT CODE
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T		
1		10S	LOAD CAPSULE												X	X								1	
2		10S	PIERCE SEPTUM					X							X	X								2	
3		20 MIN	COLLECT SAMPLE	X				X							X		X							3	
4		20S	CLEAR CAPSULE					X							X									4	
5		10S	REMOVE NEEDLE												X									5	
6		10S	TRANSFER CAPSULE													X								6	
7		10S	PIERCE SEPTUM						X															7	
8		60S	APPLY NUTRIENT		X			X		X														8	
9		20S	CLEAR CAPSULE					X		X														9	
10		10S	REMOVE NEEDLE					X																10	
11		10S	TRANSFER CAPSULE													X								11	
12		10S	PIERCE SEPTUM							X	X													12	
13		0.5, 3 MIN	UREA CLEANUP					X		X	X	X	X											13	
14		3, 5 MIN	H ₂ O CLEANUP					X		X	X	X		X										14	
15		20S	CLEAR CAPSULE					X		X	X	X												15	
16		2 HR.	2 HR. INCUBATION								X							X						16	
17		10S	REMOVE NEEDLE																					17	
18																			X	X	X	X		18	
TOTAL DWELL TIME				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T		

TOTAL DWELL TIME

AUTOMATIC PROGRAM SEQUENCE - Table 8

DRUM SEGMENT GUIDE



18 STEP
SINGLE TIME-
BASE CHART

STEPPER # 2

11-21-72

18 STEP SINGLE TIME- BASE CHART			STEPPER # 2 11-21-72																							
STEP NUMBER	CONTROL TO ADVANCE	DWELL TIME IN SECONDS	DESCRIPTION OF OPERATION																					NO OR NC CONTACT	CIRCUIT CODE	
				INDEX	PUMP # 4	CYLINDER # 4	PUMP # 3	CYLINDER # 5	3V2B - VALVE	VALVE 4V1	PUMP # 5	BASLINE RELAY	OUTPUT RELAY	SHORT OUTPUT RELAY	PUMP # 6	PUMP # 7			ENERGIZE STEPPER 1	ENERGIZE STEPPER 2						
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			
				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T			
1		10S	TRANSFER CAPSULE	X																					1	
2		10S	PIERCE SEPTUM			X																			2	
3		3 MIN.	UREA WASH		X	X	X				X						X								3	
4		10S	REMOVE NEEDLE		X						X						X								4	
5		10S	TRANSFER CAPSULE	X	X						X						X								5	
6		10S	PIERCE SEPTUM		X			X			X						X								6	
7		10S	REACTION		X			X	X								X								7	
8		5 MIN.	REACTION CON'T					X	X					X	X										8	
9		30S	H ₂ O WASH					X	X		X						X	X							9	
10		20S	CLEAR CAPSULE					X	X								X								10	
11		10S	REMOVE NEEDLE														X								11	
12																									12	
13																									13	
14																									14	
15																									15	
16																									16	
17																						X	X		17	
18																									18	
TOTAL				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T			

TOTAL
DWELL TIME

AUTOMATIC PROGRAM SEQUENCE - Table 9

DRUM SEGMENT GUIDE

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

3.6 Operation in a Simulated Zero-g Environment

The ability of the instrument to operate in a Zero-g environment was demonstrated by operating the instrument in a manual and automatic mode at 90° and 180° (upside down) to the normal plane (see Figure 19). The results summarized in Table 5, indicate that the data are comparable to those obtained on operating the instrument in the normal horizontal plane. Thus, in one series of experiments (i.e., first four runs in Table 5), the average number of cells/ml required to produce a net signal of one volt when operating the instrument in a normal horizontal plane was 107 cells (average of 118 and 95 cells/ml/volt) and 105 cells (average of 133 and 77) when operating the instrument upside down or on its side.

3.7 Demonstration of Instrument

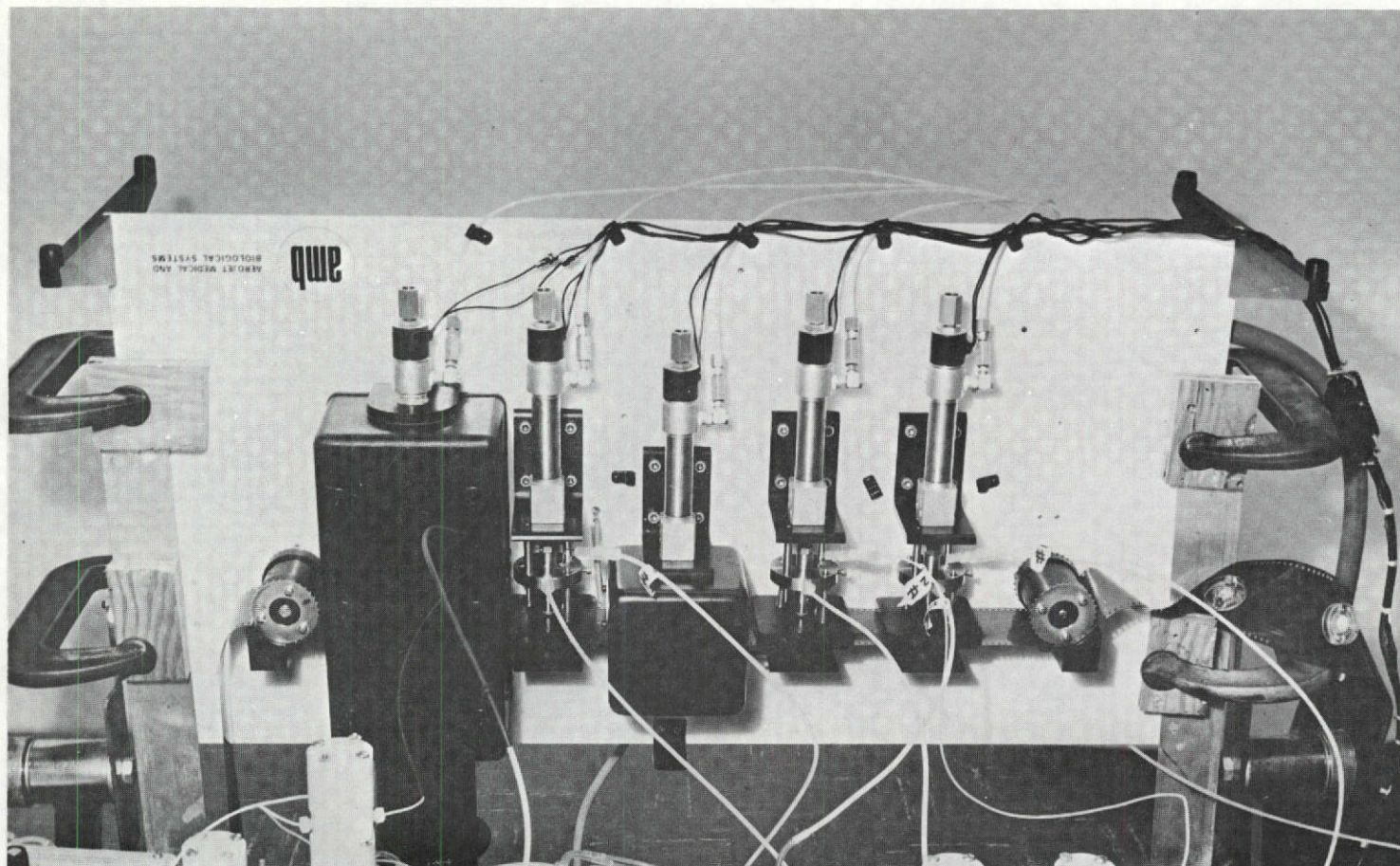
The tests indicated in Table 10 were demonstrated to DCAS's representative* with the results summarized in Table 11.** The objectives of this test series were 1) to demonstrate the total and viable cycles with E. coli using a fully automatic mode in a hands-off operation and 2) to demonstrate the Zero-g capability of the Processing Station Assembly.

The results obtained in this test series may be summarized as follows:

1. The signals obtained on operating the instrument in an automatic mode, on its side and upside down are comparable to those obtained in a normal mode.

* Defense Contract Administrative Service.

** To enable the tests to be run within a reasonable time period, 60 ml volumes of both sample and water controls were processed, instead of the 400 ml normally used.



AMB WATER MONITOR
OPERATED IN INVERTED
POSITION (180°)

Figure 19

Table 10

DEMONSTRATION OF AMB WATER MONITOR TO
DCAS REPRESENTATIVES

A. PROGRAM OBJECTIVES

1. As stated in para. 3.2.1 of Contract Work Statement - "to evaluate instrument for sensitivity toward E. coli and one additional aerobic organism using an optimized viable and nonviable cycle."

2. As specified in para. 3.2.3 of Contract Work Statement - "to design, fabricate and checkout a fully automatic tape cassette bacteria detection and sampling system based on the filter - capsule detection system. This unit is to be a fully functional prototype design, capable of zero-g operation."

B. APPROACH TO BE USED

1. Viable and nonviable cycle demonstrated with E. coli.
2. Fully automatic features demonstrated by hands-off operation.
3. Zero-g capability demonstrated by operating instrument horizontally, sideways and upside down.

C. TESTS TO BE PERFORMED

<u>Test No.</u>	<u>Type of Test</u>
1	Water Blank (Control) - Manual (Horizontal)
2	Total Cycle - Manual (Horizontal)
3	Total Cycle - Automatic (Horizontal)
4	Total Cycle - Automatic (On Side)
5	Total Cycle - Automatic (Upside Down)
6	Viable Cycle (Unincubated Control)* - Automatic (Horizontal)
7	Viable Cycle (Incubated) - Automatic (Horizontal)

*The water sample in the unincubated control is processed in the same manner as the incubated sample except that the actual incubation is omitted in the former.

Table 11

TEST RESULTS OF INSTRUMENT DEMONSTRATION

Test [*] No.	Test Sample	Operational Mode	Cycle	Max. Signal Volts ^{**}	Net Signal	Comment	Ref. Data Sheet
1	Water Blank	Manual (Horiz)	Total	4	-	-	66
2	8x10 ⁵ <u>E. coli</u>	Manual (Horiz)	Total	1	-	Reagent line blockage	66
2 (rep) [‡]	8x10 ⁵ <u>E. coli</u>	Manual (Horiz)	Total	21	17	-	66
3	8x10 ⁵ <u>E. coli</u>	Auto (Horiz)	Total	25	21	-	66
5	8x10 ⁵ <u>E. coli</u>	Auto (Upside Down)	Total	19	15	-	66
6	2x10 ⁵ <u>E. coli</u>	Auto (Horiz)	Viable (Unin- cubated) ^{***}	10.5	6.5	Possible contamin. of nutrient with Urea	67
7	2x10 ⁵ <u>E. coli</u>	Auto (Horiz)	Viable (Incub- ated) ^{***}	7.5	-	Light Leak	67
4	8x10 ⁵ <u>E. coli</u>	Auto (On Side)	Total	7.3	3.3	-	67
6 (rep)	2x10 ⁵ <u>E. coli</u>	Auto (Horiz)	Viable (Unin- cubated) ^{***}	6	3	-	68
7 (rep)	2x10 ⁵ <u>E. coli</u>	Auto (Horiz)	Viable (Incub- ated) ^{***}	12.5	-	-	68
4 (rep)	8x10 ⁵ <u>E. coli</u>	Auto (On Side)	Total	29	26	-	68
1	Water Blank	Auto (Horiz)	Total	3	-	-	68

* Test numbers correspond to those indicated in Table 10.

** Signal maximum appearing at least 5 seconds after Premix turned on.

*** Incubated 2 hours at 35°C + 1°C; the viable (unincubated) sample serves as a control for the incubated sample. The protocol for the unincubated viable sample is the same as a "Total" cycle.

‡ Test repeated.

2. Tests Nos. 4, 6 and 7 (Tables 10 and 11) failed to produce satisfactory signals in the first attempt but succeeded in the second. There is evidence to indicate (see Data Sheet No. 67), that failure to observe an increased signal on incubation the first time may have been due to the presence of traces of Urea in the nutrient line. No explanation can be offered to account for the lower than expected signal in the total cycle initially for Test No. 4.

3. Complete hand-off operation was not always achieved with problems of needle plugging, incomplete piston travel, capsule and line leakage being evident in some of the runs. When a malfunction occurred, the cycle was interrupted, the repair made and the automatic operation continued to give the signals indicated in Table 11. The malfunctions and manual assists that did occur were recorded by DCAS inspectors and will appear in a separate report to be issued by them.

Section 4

LABORATORY SUPPORT STUDIES

As part of the laboratory support effort, a number of studies were carried out in the following areas:

1. Evaluation of various parameters required for optimizing the capsule design.
2. Protocol development utilizing a prototype capsule.
3. Capsule pretreatment to minimize spurious signals.
4. Elimination of cross-contamination between stations.
5. Definition of reagent shelf life.

The results of these studies are described briefly below:

4.1 Establishing Design Parameters of Prototype Test Capsule

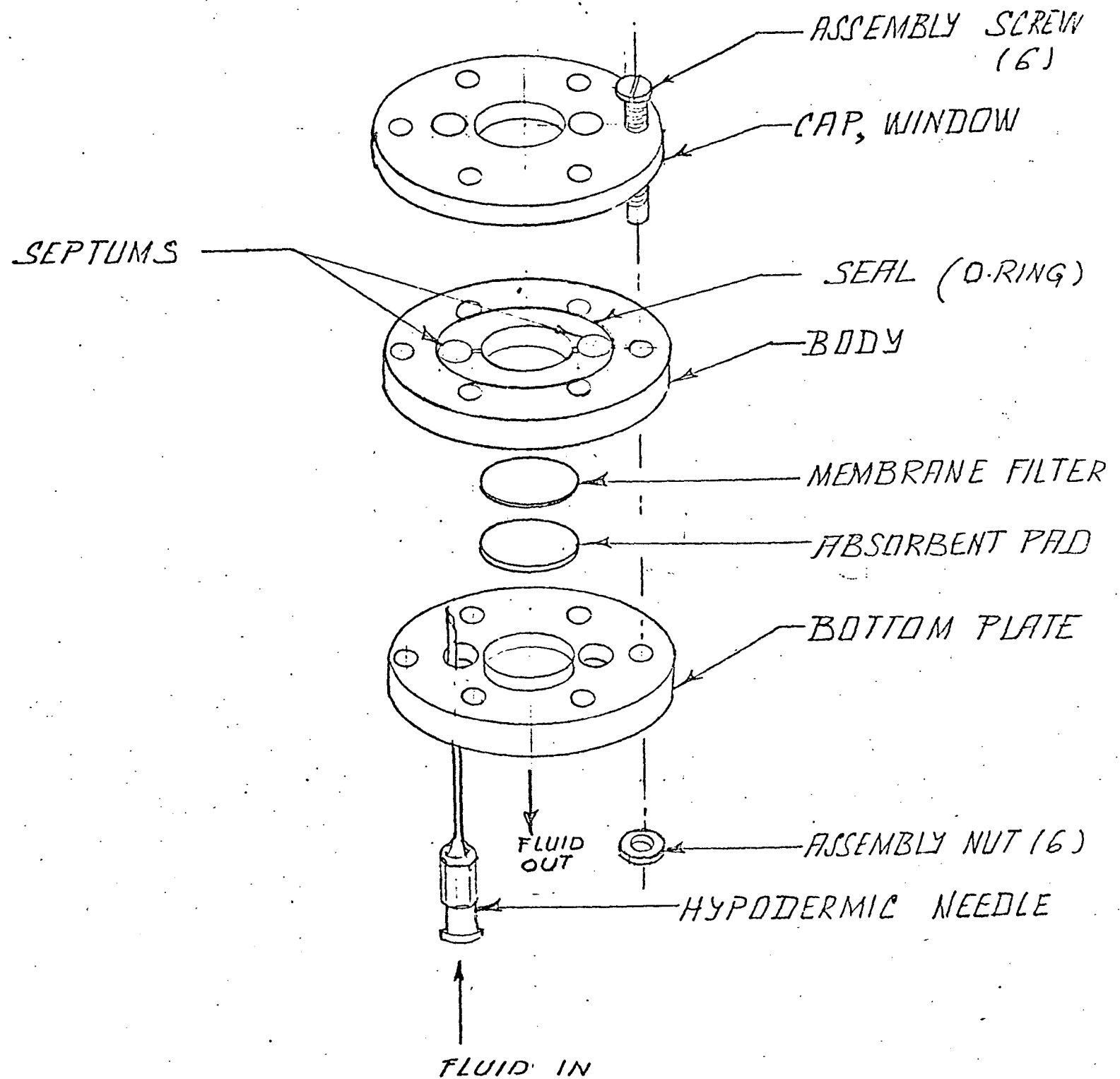
In the initial study, a prototype test capsule (shown in Figure 18) was used for evaluating a number of parameters required for establishing the optimum capsule design.

Prior to construction of this prototype capsule, a number of plastic components were evaluated for reagent compatibility, transmissivity and inhibitory effect on bacterial growth. The results of these and other tests are summarized below:

1. Transmissivity

Since the light generated on reaction is to be viewed through a plastic window (0.031" thick) in the capsule, the transmissivity of various candidate plastics was evaluated. The results before and after exposure to luminol-H₂O₂ reagent (Premix) or to autoclaving * are shown below:

* The use of autoclaving was initially considered as a means of sterilizing the capsule after assembly.



TEST CAPSULE
(SCHEMATIC)

Figure 20

Plastic	Thickness	% Transmission at 430 m μ ***		
		Before Exp. to Premix	After Exp. ** to Premix	After Autoclaving
Plexiglas †	0.125"	91	90	67
Kel-F	0.30"	87	86	87
Lexan	0.30"	81	83	84
Unplast. PVC	0.008"	82	-	79
Unplast. PVC (Blue Tint)	0.005"	81	-	58

(Refer to Data Sheets Nos. 1 through 5)

Except for the significant loss of optical clarity on autoclaving, the transmission characteristics of Plexiglas before and after exposure to reagent appear excellent. For sterilization of the capsule after assembly, pretreatment with Premix reagent was used. This strong caustic solution is an effective sterilizing medium.

2. Reagent Compatibility

The compatibility of candidate plastics with luminol H_2O_2 (Premix) reagent was evaluated by soaking these materials in Premix for 2.5 hours at ambient and then observing the signal obtained with a single suspension of E. coli. **** Premix without plastic was used as a control. A chemiluminescence device^o developed by AMB for commercial use was employed in this test. The results obtained are shown below:

** Exposure to Premix was for 2 hours at ambient.

*** Wavelength of chemiluminescent emission

**** 3/4" square of plastic soaked in 20 ml Premix.

† A direct measurement of an actual window cap (0.031" thick Plexiglas) of the final capsule design indicated a transmissivity of 92% at 430 m μ .

^o In this device a sample stream (at 8.25 ml/min.) and Premix reagent (at 3.0 ml/min.) are brought together in a glass reactor and the integrated light generated over an 8-second interval recorded on a Nixie-tube meter.

	<u>Integrated Signal, Volts</u>	<u>Average, Volts</u>
Premix without Plastic (control)	9.75, 9.86, 9.62	9.74 \pm .12**
Premix with Plexiglas	9.14, 9.14, 9.29	9.19 \pm .09
Premix with Lexan	9.91, 9.23, 9.41	9.52 \pm .29
Premix with Kel-F	8.30, 9.20, 8.90	8.80 \pm .56
Premix with Unplast. PVC (untinted)	9.30, 9.30, 9.03 (Refer to Data Sheet No. 6)	9.21 \pm .16

The data shown above indicate that based on a three sigma variation, none of the plastics tested have a significant effect on the chemiluminescence signal and all would be considered acceptable. Plexiglas was the material selected since it was available in the sheet thickness required for machining the test capsule.

3. Effect of Capsule Components on Bacterial Growth

Candidate rubber and plastic materials which might be utilized in the capsule were evaluated for their possible inhibitory effect on bacterial growth during the incubation step of the viable cycle.

The experimental procedures consisted of the following:

Prescribed materials were sterilized by autoclaving in dextrose broth (12 ml) for 15 minutes at 15 psi (250°F). To each broth tube an inoculum of E. coli was then added to a concentration of 6.7×10^5 cells/ml. The optical density (O.D.) of the broth culture was determined before and after a 4-hour incubation at 37°C. Bacterial counts were then made of the high and low O.D. values.* The results are tabulated below:

* Bacteria prestained with protein specific dye and then counted under the microscope.

** Standard Deviation.

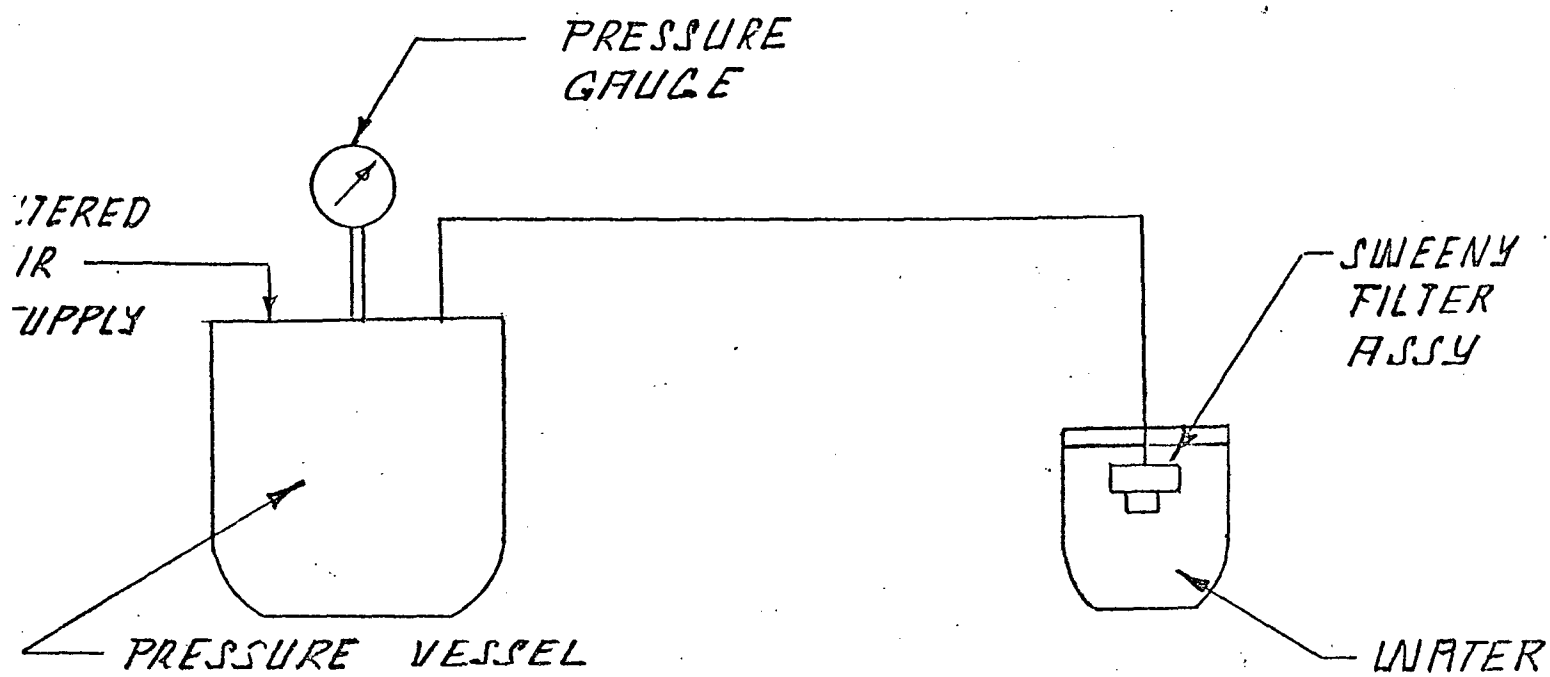
Material	Optical Density		Total Cell Count (Incubated Sample) cells/ml
	Unincubated Sample	Incubated 4 hrs. at 37°C	
Broth	0 (set)	0.22	-
Plexiglas	0.01	0.21	7.9×10^6
Kel-F	0	0.23	-
PVC (Blue Tint)	0	0.22	-
PVC (Untinted)	0	0.31	8.7×10^6
Lexan	0	0.25	-
Neoprene	0.04	0.22	-
Viton	0.03	0.27	-
Silicon Rubber (Nat.)	0.01	0.27	-
(Refer to Data Sheet No. 7)			

The bacterial cell counts for all incubated samples including the control, had increased from an initial value of 6.7×10^5 cells/ml to 8.3×10^6 ($\pm 0.4 \times 10^6$) after incubation. Since the extent of variation between different samples is of the order of only $\pm 5\%$ of the mean value, or well within the experimental error (i.e., $\sim \pm 20\%$) for this type of measurement, it may be concluded that none of the above materials inhibit the growth of the test organism.

4. Bubble Point Determination

Using a Millipore pressure vessel with attached Swinny filter assembly with membrane filter support and pad, ** (see Figure 21), the bubble point was determined after pretreating the filter with water or nutrient solution. The results were as follows:

** Filter -- Gelman Acropor AN 450 (13 mm diameter; 9 mm diameter effective filtering diameter). Pad -- Millipore (AP 100 1300).



BUBBLE POINT APPARATUS

Figure 21

Sample	Bubble Point*	
	Slow Air Release	Rapid Air Release
Water	27 psig	30 psig
Nutrient Solution	20 psig	22 psig

(Refer to Data Sheet No. 8)

5. Filter Support Requirement

Since the processing fluids are to be pressure-filtered through the membrane filter, tests were run to determine whether a supporting absorbent pad and/or screen would be required. The results shown in Table 12 indicate that (a) a pad is required, a screen is not, (b) flow can be in either direction without producing objectionable distortion of the filter membrane. Approximately 7 minutes would be required to filter a 400 ml sample through the assembly.

Initial studies (Table 13) on water extractables of the filter-absorbent pad combination indicated the presence of a water soluble component could be removed by passing 40 ml of filtered distilled water through the filter-pad assembly. However, further studies indicated that a water insoluble residue (probably dead bacteria trapped in the matrix of the absorbent pad) still remained after the water treatment which caused the pad to luminesce on contact with the luminol-hydrogen peroxide reagent. Even washing the pad with 4M Urea (a procedure found effective in rendering the membrane filter, which is considerably thinner, nonluminescent) failed to reduce the luminescence to an acceptable level. Other filter supports investigated (i. e., Millipore's AP20 and AP25 microfiber glass discs with an acrylic binder) behaved similarly.

* Tank pressure indicated.

Table 12

EFFECT OF PRESSURE FILTRATION ON
VARIOUS FILTER COMBINATIONS

Filter Arrangement	Tank Pressure	Filtration Time 400 ml*	Comments
Filter on top of pad	30 psig	7 min.	No objectionable distortion of membrane
Filter + pad + screen	30 psig	8.5 min.	" " "
Filter without pad	30 psig	filter bursts	--
Pad on top of filter	30 psig	6 min.	No excessive distortion

* Filtered-distilled H₂O

(Refer to Data Sheets Nos. 9 through 13).

Table 13

CHEMILUMINESCENCE OF WATER EXTRACTABLES FROM
MEMBRANE FILTER-ABSORBENT PAD COMBINATION*

Procedure: Sterile distilled water passed through filter-pad assembly in a Swinny holder, and successive 10 ml increments of effluent tested for chemiluminescence in an AMB Chemiluminescence Flow Device.

	<u>Average Chemiluminescence Signal, Volts**</u>
<u>Water Control (Before Filtration)</u>	2.03
<u>Water After Filtration</u>	
1st 10 ml	3.07
2nd 10 ml	3.55
3rd 10 ml	2.50
4th 10 ml	1.92

* Gelman Acropor AN 450 membrane filter; Millipore cellulose pad (AP10-013-00).

** See backup Data Sheet No. 14.

The problem was eliminated, however, by use of a stainless steel support screen*in place of the absorbent pad. Preliminary results (Table 14) indicated that this combination (i. e., membrane filter and stainless support screen) produced an acceptable reagent blank. This combination is also dimensionally compatible with the present capsule design.

6. Conclusions

Based on the foregoing studies, Plexiglas was selected for construction of the capsule body since it did possess satisfactory reagent compatibility, transmissivity and did not effect bacterial growth. Kel-F or Lexan could serve as backup plastics.

4.2 Studies with Capsule Prototype

The reuseable test capsule, shown in Figures 22 through 25, was fabricated using Plexiglas. It contains two septums instead of the intended one in order to allow for greater flexibility in protocol development.

Utilizing this prototype capsule, studies were conducted to (a) evaluate the extent of leakage which might occur on pressure-filtering a 400 ml water sample through the capsule assembly and (b) evaluate the sensitivity toward E. coli. The results obtained in each of these areas are described below.

4.2.1 Leakage Studies with Capsule

Initial trials (Table 15) on pressure-filtering a 400 ml water sample through the test capsule indicated some leakage, principally around the sides of the rubber septum, as well as through repeated punctures of the septum. This deficiency was corrected by the following design modification:

1. The diameter of the septum hole was decreased from 0.213" to 0.180". This created enough compression on the septum to eliminate leakage even after repeated (5) punctures (see Table 15).

2. The thickness of the center (body) plate of the test capsule was increased from 0.125" to 0.250" to enable the compressed rubber septum to expand on top without obstructing the liquid flow.

* Millipore XX30 012 03

Table 14

CHEMILUMINESCENCE STUDY WITH FILTER-SCREEN
COMBINATION IN PROTOTYPE CAPSULE

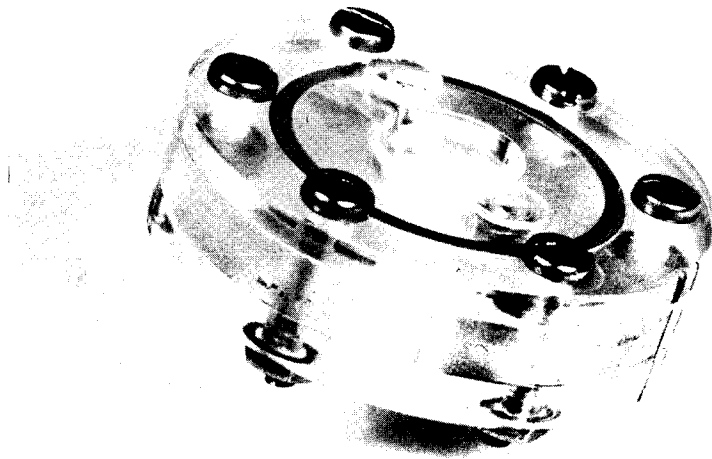
Procedure: Various concentrations of E. coli (contained in 5 ml of sterile distilled water) were deposited on a membrane filter-metal screen combination in a prototype capsule. Luminol-H₂O₂ reagent was then brought in and the peak voltages recorded.*

Run No.	Challenge	Sample	Signal, Volts		
			Noise***	Net Signal	Overall Net Signal**
1	Water-Reagent Blank	2	2	0	--
2	1x10 ⁵ <u>E. coli</u>	12.5	6	6.5	6.5
3	2x10 ⁵ <u>E. coli</u>	26	6.5	19.5	19.5
4	5x10 ⁵ <u>E. coli</u>	>48 (Sat.)	6	>42	>42

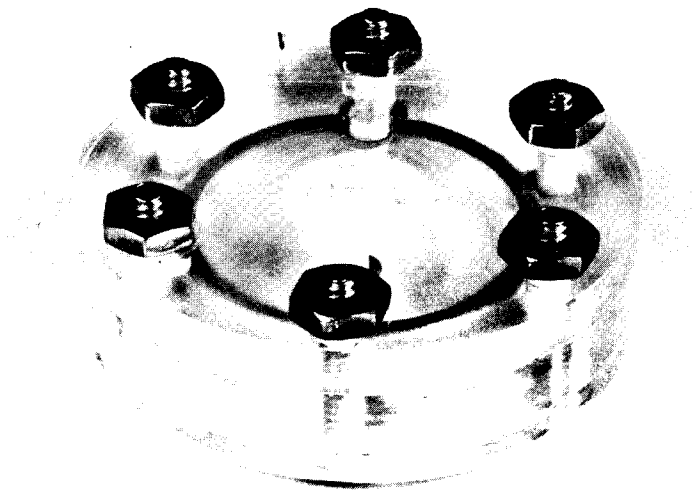
* See Data Sheet No. 15.

** Overall net signal is obtained by subtracting net signal for the water-reagent blank from the net sample signal (e.g., at 2.5x10⁵ E. coli, the overall net signal is 19.5-0=19.5 volts).

*** Noise refers to the equilibrium signal produced by interaction of the filter with the reagent.



TOP VIEW



BOTTOM VIEW

Figure 22 TEST CAPSULE
(2" Diameter; 9/16" Thick)



F.K. MULLIGAN 3-10-72

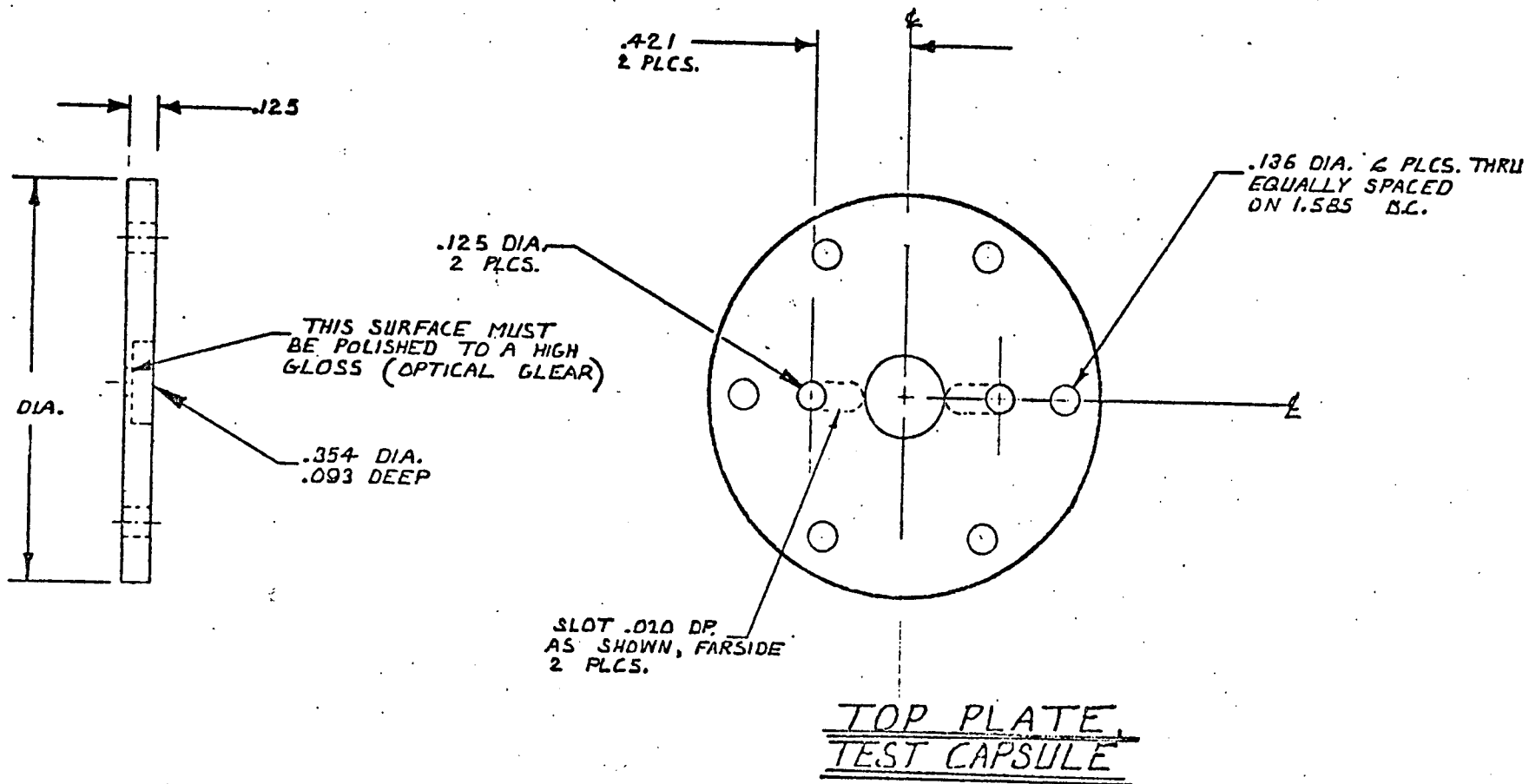


Figure 24

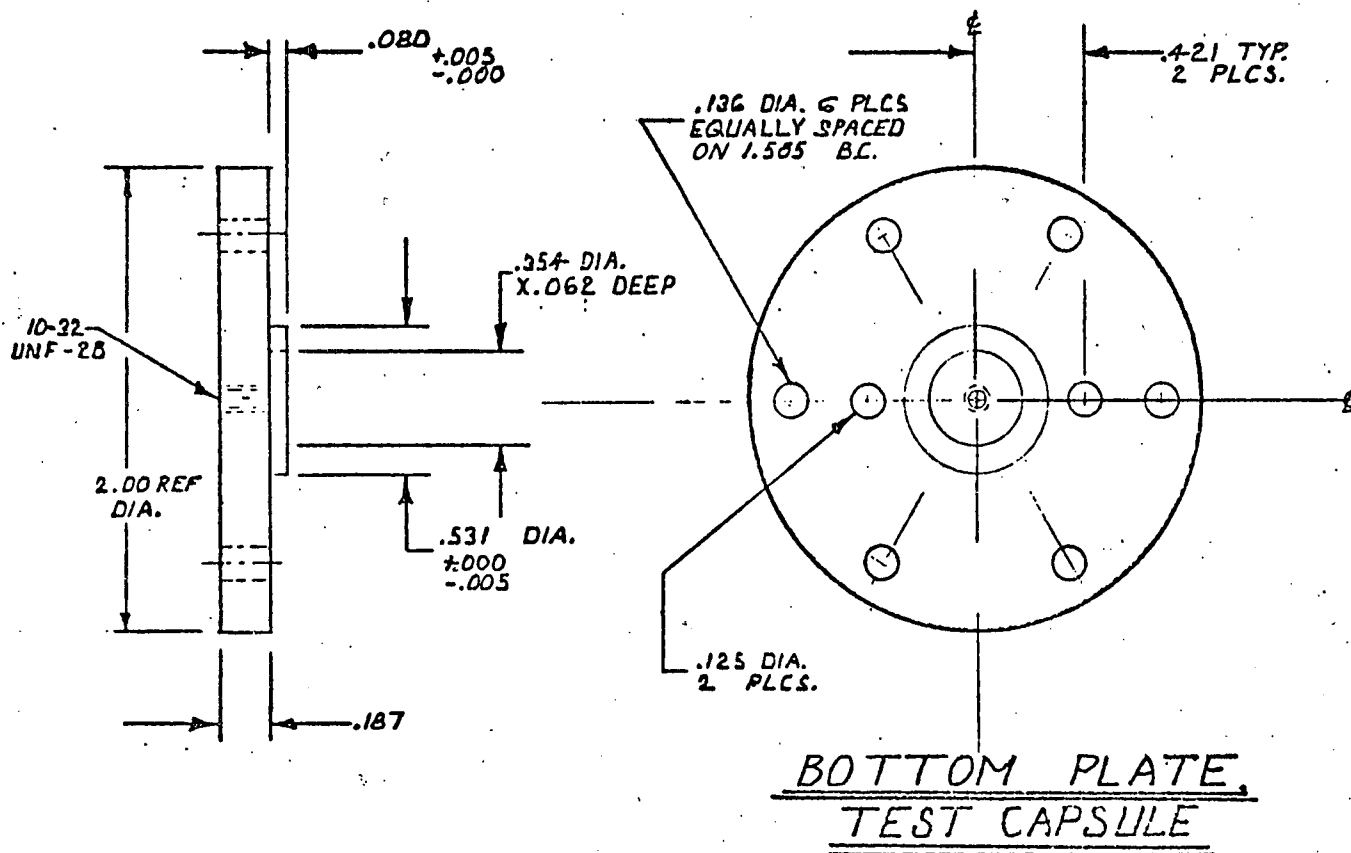


Figure 25

Table 15

LEAKAGE STUDIES - TEST CAPSULE

Ref. Data Sheet No. ***	Tank Pressure (psig)	Test Capsule	Type Septum	Needle Gauge	Filt. Time/Vol.	Leakage
14 A	30	Thin Body Plate *	Hamilton (Larger Septum Hole)**	26	8 min/120 ml	Leakage both septa
15 A	"	"	"	18	36 min/400 ml	Leakage around punctured septum
16 A	"	"	Hamilton (Reduced Septum** Hole)	26	22.5 min/400 ml	Slight leakage opposite septum †
17 A	"	"	"	26	23.5 min/400 ml	Slight leakage (~ 0.3 ml) opposite septum
18 A	"	Thicker Body Parker Plate*	(longer septum)	26	18 min/400 ml	Leakage (~ 1 ml) opposite septum (crack in center body plate)
19 A	"	"	"	18	13 min/400 ml	Leakage (~ 0.7 ml) pierced septum (crack in center body plate)
20 A	"	"	"	26	20 min/400 ml 30 min/570 ml	No leakage

* Center body plate of capsule increased in thickness from 0.150" to 0.250."

** Diameter of septum hole decreased from 0.213" to 0.180."

*** Refer to Appendix E.

† Septum without needle

3. Hamilton's silicone rubber septum (Microsep F-138 Septum) was replaced by a silicone rubber septum fashioned from a Parker silicone O-ring (#70 Shore). The latter provided better sealing for the present configuration.

The data (shown in Table 15) indicate that 20 minutes are required to pressure-filter (at 30 psi tank pressure) a 400 ml water sample using a 26-gauge hypodermic. No leakage was evident even on puncturing the rubber septum four times prior to beginning the test (5 punctures in all).

4.2.2 Sensitivity Toward E. coli*

1. Unincubated Sample

Several tests were made to determine the effect on sensitivity of purging the capsule with air (to remove the Urea** from the cavity) prior to reaction with the Premix reagent. The results shown in Table 16 indicate somewhat higher net signals with this procedure compared to one in which the Urea in the capsule was not removed prior to mixing with reagent. However, the magnitude of the observed increase was not sufficient to offset the increased complexity of the system design that would be required and consequently the air purging feature was not used.

Additional studies were performed to determine the sensitivity that might be achieved using the entire processing protocol (i.e., sample concentration, adding nutrient, washing with 4M Urea and reaction with Premix). In these runs the bacteria were contained in only 1 ml instead of the 400 ml intended to be used eventually. The results of several runs

* The PMT assembly from the previous contract (NAS 9-11644) was modified to accept the prototype capsule. The Premix mixture was flowed into the capsule at 1.35 ml/min. through a hypodermic needle inserted in the rubber septum of the capsule. There was no partial vacuum utilized to exhaust the liquid passing through the filter membrane.

** In the proposed procedure, a Urea wash is passed through the capsule prior to reaction with luminol-hydrogen peroxide (Premix) at the readout station.

Table 16

EFFECT OF DRY/WET CAPSULE FILTER ON
CHEMILUMINESCENCE SIGNAL

Procedure: E. coli (in 1 ml) deposited on filter in capsule and then washed with 4 M Urea. In the "Dry" procedure, the residual Urea in the capsule was blown out by forcing air through the capsule prior to reacting with Premix reagent. In the "Wet " procedure, the Urea was not removed prior to reaction with Premix. The peak voltages were recorded.*

	Net Signal (Volts)	
	<u>Wet Capsule</u>	<u>Dry Capsule</u>
Water Blank	0, 1	0
1×10^5 <u>E. coli</u>	3.5	6
3×10^5 <u>E. coli</u>	17.5, 21.5	25

* See Data Sheets Nos. 16 and 17.

(Table 17), shown plotted in Figure 26, indicate that a total of 5×10^4 E. coli produce an overall net signal of 3 volts. If contained in a 400 ml sample, this challenge of 5×10^4 cells would correspond to 125 cells/ml.

2. Incubated Sample

Two procedures were compared for incubating the bacteria in the prototype test capsule. One entailed introducing the nutrient through the capsule bottom (i. e., waste exit) in sufficient quantity to wet the filter and the other, introducing the nutrient via the hypodermic piercing the rubber septum. Following a two-hour incubation, 50 ml of 4M Urea were passed through the capsule (to remove nutrient) and then reacted with luminol-hydrogen peroxide in front of a photomultiplier.

The results, summarized in Table 18, indicate a slight if significant difference in the resulting net signal between the two methods. Inasmuch as some bacteria could be lost (through venting needle in septum) by bringing the nutrient up through the center of the capsule, the procedure which entails adding the nutrient through the needle was finally selected.

4.3 Finalized Capsule Design

Based on the foregoing studies with the prototype capsule, the capsule design shown in Figures 1, 27 and 28 was selected for fabrication of a mold. This mold was used for producing a sufficient number of capsules required for optimization of process variables and the evaluation of instrument performance (see Section 3 for details). The procedure used for assembling the capsule components is summarized in Table 1.

4.4 Capsule Pretreatment

The capsule pretreatment found effective in eliminating spurious signals and rendering it sterile involved the following steps (see Table 19).

1. Filter membrane is soaked in 4M Urea for 30 minutes.
2. Filter is then passed through two rinses of freshly distilled water.
3. Filter is air-dried and assembled in capsule.
4. Assembled capsule is placed in a vacuum desiccator (25" Hg) for one-half hour to remove trace organic volatiles.

TABLE 17

CHEMILUMINESCENCE SIGNAL OF UNINCUBATED
WATER SAMPLES CONTAINING E. coli

Procedure: 1 ml E. coli suspensions deposited in prototype test capsule, washed with nutrient (without added hemin) and then with 4 M Urea. Premix reagent then passed through the capsule and the maximum voltage recorded. Water-reagent control handled similarly.

	Signal, Volts				Ref. Data Sheet No.
	Sample	Noise	Net Signal	Overall Net Signal	
Water-Reagent Blank	3	2	1	--	20
5×10^4 <u>E. coli</u>	7	3	4	3	20
1×10^5 <u>E. coli</u>	11.5	2.5	9	8	20
2×10^5 <u>E. coli</u>	20	5.5	14.5	13.5	21
Water-Reagent Blank	5.5	4.5	1	--	19
1×10^5 <u>E. coli</u>	13.5	6	7.5	6.5	19
3×10^5 <u>E. coli</u>	35	6	29	28	19
Water-Reagent Blank	4	3.5	0.5	--	18
1×10^5 <u>E. coli</u>	17	6	11	10.5	18

SIGNAL RESPONSE OF E. coli

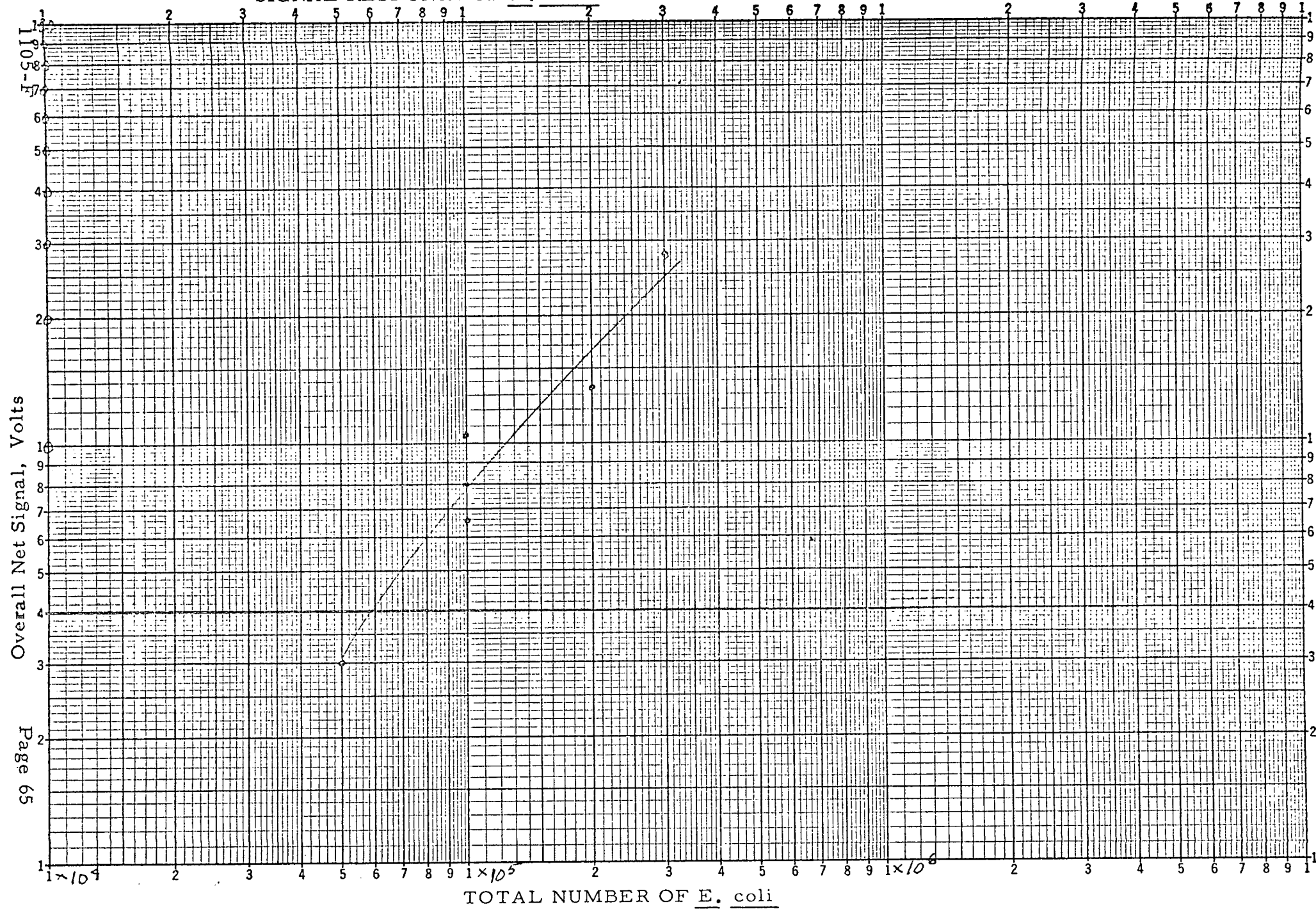


Figure 26

Table 18

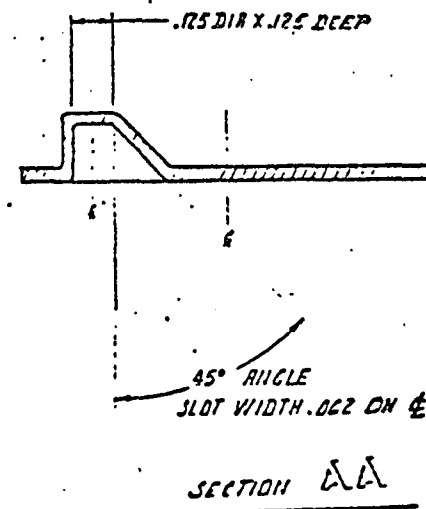
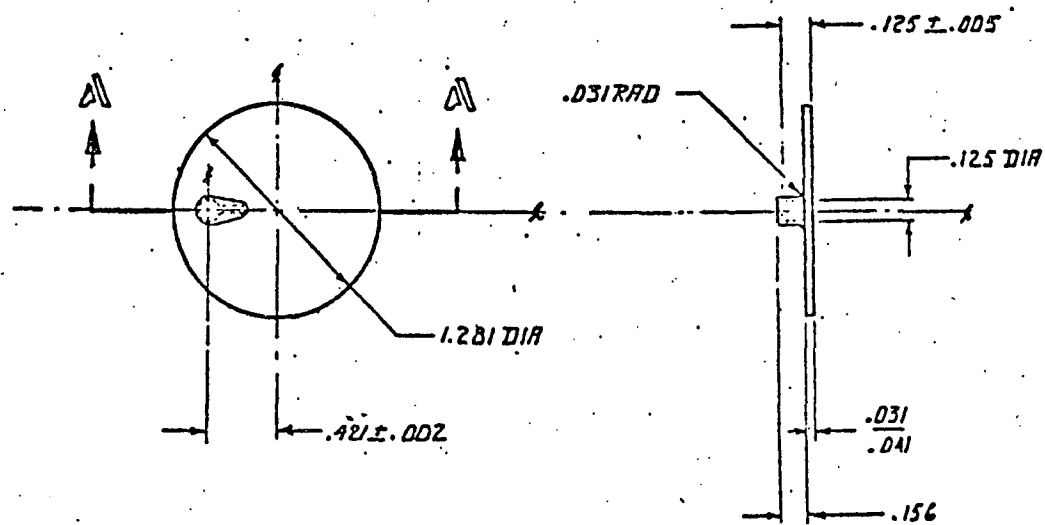
CHEMILUMINESCENCE SIGNAL OF INCUBATED
WATER SAMPLES CONTAINING E. coli

Procedure: 1 ml E. coli suspension deposited in prototype test capsule, nutrient added through hypodermic needle or center hole. The capsule was then incubated two hours, 37°C. Capsule washed with 4M Urea and then reacted with Premix in normal manner. The maximum voltage was then recorded.*

Signal, Volts

	<u>Nutrient Added through Needle</u>			<u>Nutrient Added through Bottom Center Hole</u>		
	Signal	Noise	Net Signal	Signal	Noise	Net Signal
Water-Reagent Blank	4	3.5	0.5	5.5	4.5	1
1x10 ⁵ <u>E. coli</u> (uninc.)	17	6	11	13.5	6	7.5
1x10 ⁵ <u>E. coli</u> (incub.)	32	6	26	34	9	25

* See Data Sheets 18 and 19.



(Dwg. #1000172) CAP, WINDOW

Figure 27

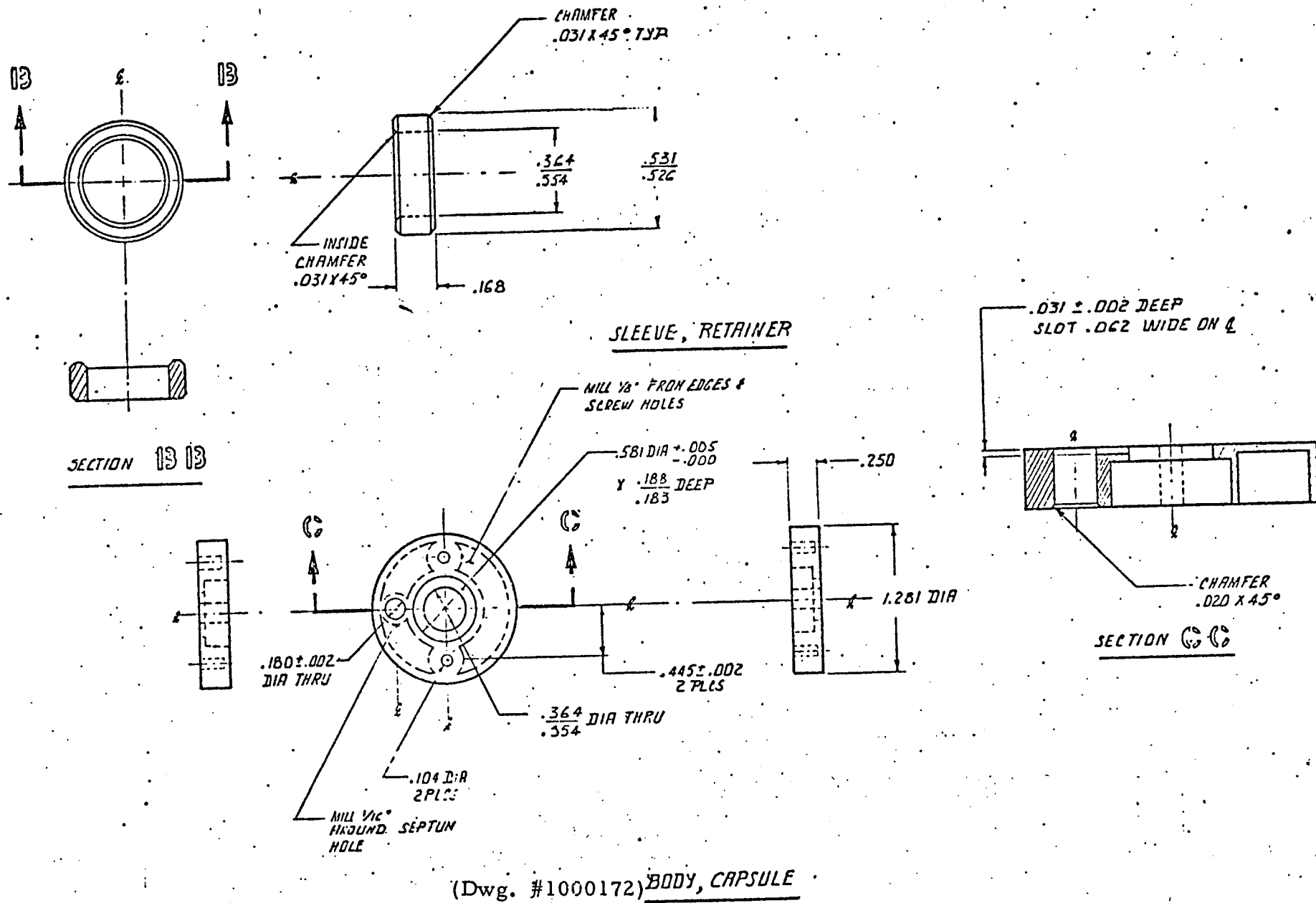


Figure 28

Table 19

CAPSULE PRETREATMENT

1. Place filters in freshly filtered (0.22 μ Millipore filter) 4M Urea for at least 30 minutes.
2. Remove filters and subject to two rinses of filtered-distilled water for at least 5 minutes.
3. Remove the filters, one at a time, with a pair of tweezers. Clamp the filter on its perimeter to avoid damaging it. Air dry filter.
4. Place the pretreated filter directly into the partially assembled capsule and secure it with the stainless steel sieve.
5. Complete assembly of the capsule.
6. Vacuum-dry the treated capsules in a dessicator for at least one-half hour.
7. Secure dried capsules on film.
8. Align the first capsule at Station #5.
9. Activate Pump #6 and Cylinder #5.
10. Turn on PMT high voltage supply and adjust to desired voltage.
11. Activate Valve 4V1 and Pump #4. Allow 2 minutes to flush old Premix out.
12. Deactivate Valve 4V1 and activate Valve 3V2A.
13. Once baseline is reached, deactivate Pump #4 and Valve 3V2A.
14. When peak levels out, activate Pump #4 and Valve 3V2A.
15. Proceed with Steps 13 and 14 until peak seen at Step 14 is below 5 volts.
16. Wash Premix pretreated capsule with filtered-distilled water by activating Valves 4V1 and 3V2A and Pump #7.
17. Once baseline is reached, deactivate Pump #7, Valve 4V1, Valve 3V2A, Pump #6 and Cylinder #5 (in that sequence).
18. Index next capsule into position and proceed with Step #9.
19. Observe pretreated capsule for any obvious leaks; reject leakers.

5. Capsule is subjected to several "stop-flow" cycles with Premix (3 to 5 being generally sufficient) at Station # 5 until the reagent baseline signal drops below 5 volts.

6. Capsule is then rinsed with filtered-distilled water on the system.

7. Capsule is air-dried (off the system) at 165°F (if capsule is to be used immediately, the air drying step can be eliminated).

During the above treatment, if the capsule shows signs of leakage, it is rejected.

The capsule treated in this manner is suitable for use in the incubated and unincubated cycles.

4.5 Cross-Contamination Study

A series of tests were performed to establish that the Urea-water post-wash at the nutrient station (Station No. 2; see Table 3 and Figure 13), is effective in preventing cross-contamination between Stations No. 1 and 2. By cross-contamination is meant the transfer of bacteria in a contaminated water sample to the nutrient supply at Station 2. Contamination of the latter could produce spurious signals in subsequent samples.

The sequential series of tests that were performed consisted of the following: (See Data Sheet No. 61).

1. A water-blank (of filtered-distilled water) which served as a control, was processed on the system using the standard protocol.

2. A capsule containing 4×10^5 E. coli was then processed in the normal manner passing through Station No. 2.

3. A waste capsule was then placed in position at Station No. 2, and then 10 ml of 4M Urea, followed by 10 ml of filtered-distilled water passed through the capsule at Station No. 2.

4. The sample station was cleansed by passage of 50 ml of 4 M Urea and 50 ml of filtered-distilled water through the inlet line.

5. A water-blank was then processed on the system using the standard protocol, except that it was incubated for 2 hours at 37°C (at Station No. 3) before washing with 4M Urea and reacting with Premix at Station No. 5.

6. A comparison of the two water-blanks (from Step 1 and Step 5), showed then to be identical (i. e., 6 volts; see Data Sheet No. 61) indicating the absence of cross-contamination.

4.6 Reagent Shelf Life

Shelf life studies were performed on the required reagents at their usable concentrations * with the following results:

1. Luminol and Hydrogen Peroxide Solutions

Luminol solution (0.82 g luminol, 49.2 g NaOH, 12.3 g EDTA per liter aqueous solution) and 1% H₂O₂ were each stored in polypropylene containers with snap-on lids at ambient temperature and at 37°C for a period of one month. They were then combined in the required concentration and checked against a single bacterial level of E. coli using an AMB Chemiluminescence Flow System. One-day old luminol and freshly prepared H₂O₂ were used as controls. The results summarized in Table 20, indicate that the chemiluminescence signals of the aged and unaged reagents agree within experimental error, indicating no significant difference. Other in-house studies conducted at AMB ** indicate that similar solutions of luminol stored for periods up to six months at ambient exhibited less than 10% variation from the mean (actual standard deviation for two test series were $\pm 4\%$ and $\pm 7\%$ from the mean; one-day old luminol was used as a control). For maximum stability of the luminol reagent access of O₂ and CO₂ must be excluded. Storage in sealed (unplasticized) polyvinyl chloride containers would provide maximum stability in this regard (the permeability of unplasticized PVC to CO₂ and O₂ is approximately 1/80 and 1/40 respectively, that of low density polyethylene). Freezing does not effect the luminol solution, but elevated temperatures (>50°C) produces significant loss in sensitivity.

* See Appendix D, Reagent Preparation.

** AMB Cell Monitor, Engineering Preliminary Design Review, 13 October, 1970.

Table 20

EFFECT OF AGING LUMINOL AND H_2O_2
ON CHEMILUMINESCENCE SIGNAL

	<u>Average** Chemiluminescence Signal, Volts</u>
Premix prepared from unaged Luminol* + H_2O_2 * (containing 5×10^5 <u>E. coli</u> /ml)	6.90 (S = \pm 0.30)
Premix prepared from Luminol and H_2O_2 aged one month at ambient (containing 5×10^5 <u>E. coli</u> /ml)	7.72 (S = \pm 0.33)
Premix prepared from Luminol and H_2O_2 aged one month at 37°C (containing 5×10^5 <u>E. coli</u> /ml)	6.93 (S = \pm 0.28)

* Luminol contained 0.82 g luminol/liter; the H_2O_2 was 1%.

**An AMB Chemiluminescence Flow System was used for making the measurement. Readings at 2 M/L gain setting; average of four readings.

Hydrogen peroxide is quite stable in the pure state (90% H₂O₂ decomposes less than 1.5% per year at 25°C). However, on dilution with water, care must be exercised not to introduce trace amounts of organics or metal ions, either of which would accelerate the decomposition of the H₂O₂.

The present study appears to indicate that 37°C (98°F) is a safe upper storage temperature for each of these reagents.

2. 4M Urea Solution

The 4M Urea was stored in a polypropylene container with snap-on lids at ambient temperature and 37°C for a period of seven weeks. A bacterial suspension in 4M Ureas was then reacted with Premix (luminol-H₂O₂) reagent in an AMB Chemiluminescence Flow System using freshly prepared 4M Urea as a control. These conditions approximate those which occur when the sample capsule containing residual 4M Urea (from the Urea wash at Station No. 4) reacts with luminol-H₂O₂ at Station No. 5. The results summarized by the data in Table 21, indicate no significant difference in chemiluminescence signal of the aged and unaged Urea solutions. Earlier studies * indicated that 4M Urea is stable for at least five weeks at ambient. The present study indicates this solution to be stable for at least seven weeks at temperatures up to 37°C.

3. Dextrose Broth

This nutrient broth was stored for one month at both ambient and 5°C in the dark and then checked for its ability to support the growth of an E. coli inoculum. Unaged dextrose broth was used as a control. Growth at 37°C (four hours incubation) was evaluated by direct microscopic count (using a protein specific stain). The extent of growth was found to be the same for all three media (i.e., multiplication factor of 2×10^2 over the original inoculum**) indicating no deterioration of the nutrient by storage at either ambient or 5°C (both in the dark). Earlier studies indicated some deterioration on storage after 5 weeks at ambient when exposed to the light. Apparently, storing in the dark prolongs the shelf life of this nutrient.

* AMB Final Report No. 1504-F, to NASA/MSC, Development of "Bacterial Sensing Instrumentation for Spacecraft Water Systems," 13 November 1970, Contract NAS 9-10432.

** Initial concentration 2.5×10^6 / ml; after 4 hours at 37°C the cell count was the same in all three tubes (i.e., 5×10^8 /ml).

Table 21
EFFECT OF AGING UREA
ON CHEMILUMINESCENCE SIGNAL

	<u>Average Chemiluminescence* Signal, Volts</u>
Unaged 4 M Urea + 7.5×10^5 <u>E. coli</u> /ml	8.68 (S = \pm 0.22)
4 M Urea + 7.5×10^5 <u>E. coli</u> /ml (Aged 7 weeks at ambient)	8.33 (S = \pm 0.29)
4 M Urea + 7.5×10^5 <u>E. coli</u> /ml (Aged 7 weeks at 37°C)	8.87 (S = \pm 0.15)

*An AMB Chemiluminescence Flow System was used for making the measurement. Reaction was with fresh Premix at 2 MA gain setting of instrument; average of four readings; S = Standard Deviation.

4.7 Effect of Anode Voltage on PMT* Output

The effect of anode voltage on PMT output was evaluated at two light levels** approximating the range of interest. The results shown in Table 22 and plotted in Figure 29 indicate marked deviation from linearity above 1400 volts at the higher light level input.

Although the particular PMT utilized was rated at 1180 volts, data generated during the course of the program ranged from 1100 - 1500 volts.

* Photomultiplier tube (PMT) used was an EMI 9635B.

** Established by using a defined light leak into the PMT light shield assembly.

Table 22

ANODE VOLTAGE VS PMT OUTPUT

A. LOW LIGHT LEVEL INPUT

<u>Anode Voltage</u>	<u>PMT Output (Volts)</u>
1100	0.13
1200	0.25
1300	0.50
1400	0.83
1500	1.35
1600	2.15
1700	3.30
1800	4.80

B. HIGH LIGHT LEVEL INPUT

<u>Anode Voltage</u>	<u>PMT Output (Volts)</u>
1100	2.30
1200	5.80
1300	12.0
1400	21.0
1500	26.5
1600	31.0
1700	34.8
1800	38.2

ANODE VOLTAGE VS PMT OUTPUT

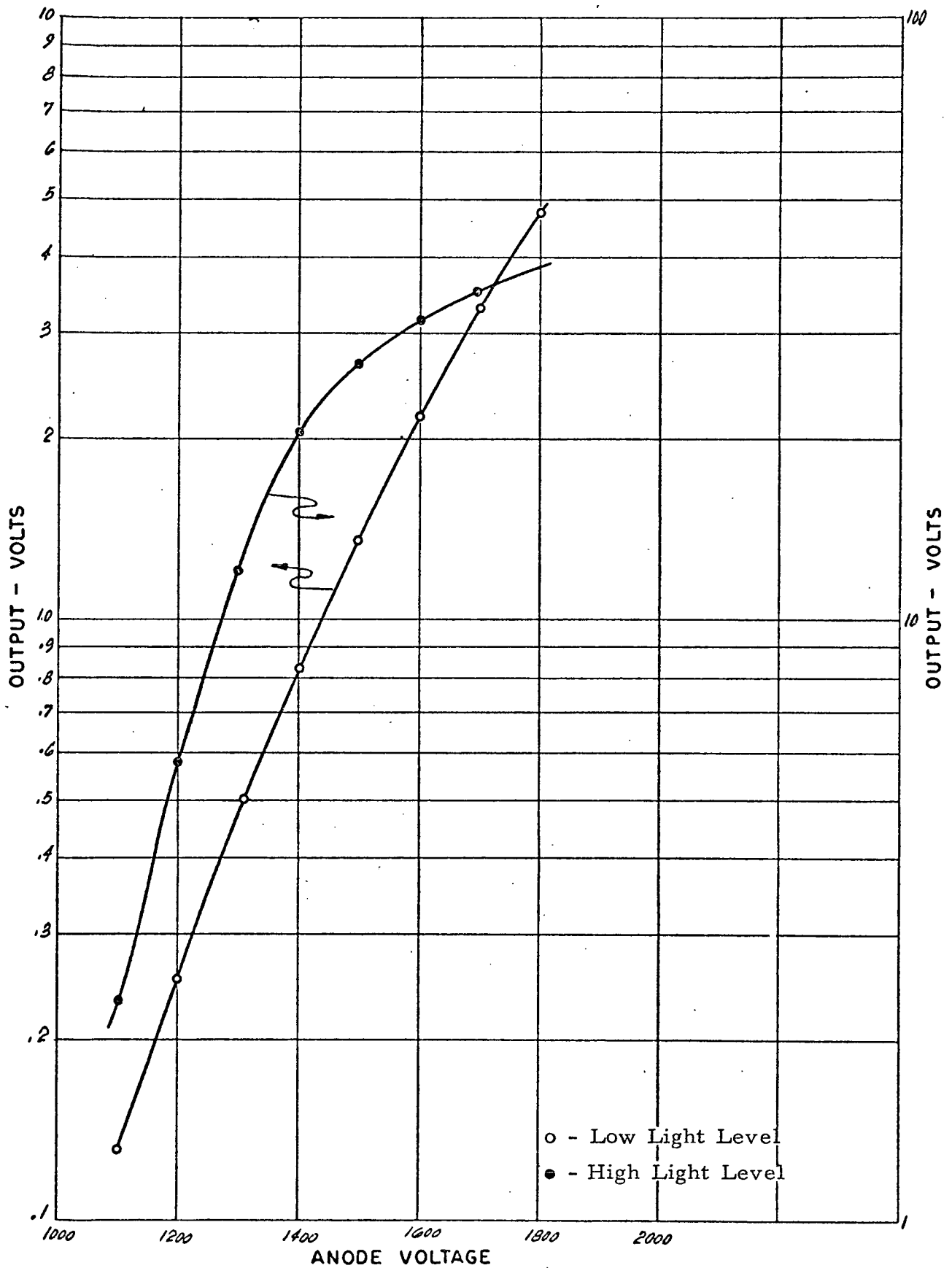


Figure 29

Section 5

CONCLUSIONS AND RECOMMENDATIONS

In keeping with the program objectives, the design, fabrication, and checkout of an automatic chemiluminescence bacteria detection system based on the filter-capsule approach have been completed. A number of problems relating to the operational reliability of the instrument have become evident, however, and these will have to be resolved before the instrument can be operated in a completely "hands-off" automatic mode with any degree of confidence. The major problems are; 1) needle plugging, 2) misalignment of capsule relative to station components, 3) light leak at Station 5, and 4) capsule leakage. The replacement of the peristaltic pumps by positive displacement pumps for reagent metering would also remove a frequent source of mechanical breakdown. The use of a solid pointed needle with a hole on the side in conjunction with a softer septum material (i.e., Viton) may eliminate needle plugging.

The Zero-g capability of the Processing Station Assembly has been demonstrated. What remains is adapting the reagent feed subsystem for Zero-g application; the use of an air-actuated "one-shot" syringe pump to deliver a discrete liquid volume would be one approach.

The sensitivity goals for the current program were ≤ 5 cells/ml (viable) and ≤ 10 cells/ml (total) for a 400 ml sample (or total bacterial challenges of 2×10^3 and 4×10^3 bacterial cells, respectively). The detection threshold* for E. coli and S. marcescens in the present program are, at best approximately 60-100 cells/ml for E. coli, and approximately 200 cells/ml for S. marcescens using the total cycle (processing time - 36 minutes), and 20-60 cells/ml for E. coli and 150 cells/ml for S. marcescens using the viable cycle (processing time - 155 minutes). There are a number of system modifications which can be introduced that would aid in approaching the sensitivity goals initially established for this system. The major changes would include the following:

* Assuming a net signal of one volt as significant and the use of a 400 ml water sample.

1. Flowing reagent across the filter instead of through it (with a minimum residence time of eight seconds in the capsule). An alternate approach would be to inject a discrete amount of Premix reagent up through the bottom center of the capsule. In either approach, a discrete amount of luminol- H_2O_2 reagent would be injected into the capsule in sufficient volume to fill the reaction cavity. To minimize liquid channeling at the readout station, residual Urea should be voided by blowing filtered air through the capsule at the previous station.

2. Better optical coupling between capsule and PMT. This might be achieved by a) bringing the capsule closer to the cathode surface, and b) use of a light gathering and reflecting shield placed around the capsule.

Other program requirements which have been met include:

1. A definition of reagent shelf life. Studies on the current and previous programs indicate that 4M Urea is stable for at least seven weeks at 37°C , nutrient broth for at least one month at ambient (in the dark), and luminol and hydrogen peroxide for at least six months at ambient or at least one month at 37°C . It is to be expected that more extensive studies will reveal a useable shelf life for all these reagents of at least six months if stored in sealed containers in the absence of air and light. An indefinite shelf life of one year or longer might be achieved (for all except the H_2O_2) by storing the reagents as dry powders and reconstituting with water just prior to use. If properly prepared (in the absence of organics or metal impurities) the H_2O_2 reagent should be stable for at least one year at ambient temperature. Additional studies are needed to evaluate the compatibility of the reagents with pump components.

2. A complete set of Level 3 engineering drawings of the instrument have been submitted to NASA.

APPENDICES

Appendix A

STARTUP PROCEDURE

Power Requirements:

95 psig Air

117 VAC

Preliminary Preparations: (See Figure 13)

1. Install appropriate plumbing.
2. Make necessary electrical (power) connections (valves, pumps, electronics, high voltage) - use common ground to avoid ground loops.
3. Connect all air lines properly and recheck before attachment to pressurized source.
4. Make recorder connections (impedance matching, calibration, chart loading).
5. Check all switch functions for proper operations, i.e. pumps, electronics, cylinders, valves. For Steps 6 - 9 prior to initial testing.
6. Flush pump tubing lines at stations #1 and #2 with 50 - 100 ml of 4M Urea. Follow with 50 - 100 ml of filter-distilled water. Then prime lines with proper fluids (sample @ station #1 and nutrient @ station #2).
7. Flush H₂O #1 and H₂O #3 lines with 25 - 50 ml of 4M Urea (recycle through valve 3V4 to waste for H₂O #1 line). Follow with another 25 - 50 ml of filter-distilled water.
8. Purge Urea #1 and Urea #2 lines for 5 minutes (recycle through valve 3V3 to waste for Urea #1 line).
9. Purge premix line for 5 minutes.
10. Fill necessary reagent supply containers daily.
 - a. Stock Luminol - 0.60 ml/min.
 - b. Hydrogen Peroxide 1% - 1.10 ml/min.
 - c. Premix Diluent-filter-distilled water - 4.36 ml/min.
 - d. Dextrose Broth - growth media - 5.4 ml/min.

Premix
Reagents

- e. 4M Urea - 3.8 ml/min @ Urea #1
6.0 ml/min @ Urea #2
- f. Filter-distilled water - 3.8 ml/min @ H₂O #1
3.9 ml/min @ H₂O #3
- 11. Check incubator temperature at steady state.
- 12. Adjust Photomultiplier Tube voltage as desired.
- 13. Check Premix reagents individual flow rates. Adjust pump or concentrations to comply with specified rates.

Appendix B

MANUAL OPERATIONAL PROCEDURE (See Figure 13)

1. RESET Stepper drums to Step 18 on Stepper #1 and Steps 12, 13, 14, 15 or 16 on Stepper #2.
2. String capsule-containing tape through each station indexing drum. Position the first capsule at Station #1 by slowly advancing tape until capsule is located directly underneath capsule seat. This is done by manually assisting Cylinder #1 up and down until capsule is seated. Switch to manual control and power on.
3. Activate cylinder #1.
4. Start waste pump (#6).
5. Activate sample pump (#1).
6. Deactivate pump #1 after desired volume is passed through capsule.
7. Deactivate pump #6 after waste line is clear of fluid (10 seconds).
8. Deactivate cylinder #1.
9. Transfer capsule to station #2 (Index).
10. Activate cylinder #2.
11. Start waste pump #5.
12. Apply nutrient with pump #2.
13. After one (1) minute, deactivate pump #2.
14. When exit line is clear of fluid (10 seconds) deactivate cylinder #2.
15. Transfer capsule to station #3 (Index). If a viable cycle is desired, activate cylinder #3 (incubator must be brought up to desired temperature and start timer. Allow additional 5 minutes for capsule to reach steady state).
16. Activate cylinder #2.
17. Activate valves 3V3 and 3V1. Apply 4M Urea through waste capsule for three (3) minutes.
18. Deactivate valve 3V3 and activate valve 3V4. Apply filter-distilled water through waste capsule for three (3) minutes.
19. Deactivate valves 3V3 and 3V1.

20. Terminate pump #5 when fluid in exit line is cleared (10 seconds).
21. Deactivate cylinder #2.
22. After incubation (viable cycle) is complete, deactivate cylinder
- #3. If running a total cycle, proceed to next step and omit steps 14 and 21.
23. Transfer capsule to station #4 (Index).
24. Activate cylinder #4.
25. Activate valve 4V1.
26. Start premix waste pump (#6).
27. Start premix pump (#4).
28. Activate pump #3 and allow three (3) minutes of continuous flow.
29. Terminate pump #3 and wait ten (1) seconds for capsule to equalize.
30. Deactivate cylinder #4.
31. Transfer capsule to station #5 for readout (Index).
32. Activate cylinder #5.
33. Secure light shield and place black cloth over shield or turnoff lights.
34. Turn on recorder (set chart speed and full scale reading as desired).
35. Switch PMT high voltage power supply on and adjust voltage as desired.
36. Simultaneously activate valve 3V2A and deactivate valve 4V1. Once recorder needle reaches five (5) volts or ten (10) seconds have elapsed, deactivate valve 3V2A and pump #4 again, simultaneously.
37. Record the maximum voltage output from peaks originating from sloping curves but not from spikes (caused by pressure differentials or undesirable chemical activity).
38. Once a baseline (change in slope of curve is minimal) is reached, activate waste pump again (#6).
39. Start water waste pump (#7).
40. Activate valves 4V1 and 3V2A simultaneously and flush for thirty (30) seconds.
41. Deactivate valve 4V1, pump #7, valve 3V2A, and pump #6 in that order.

42. Turnoff PMT voltage source.
43. Deactivate cylinder #5.
44. Proceed loading sequence at station #1 again or if a continuous tape is used, index over to station #1.

Appendix C

SHUTDOWN PROCEDURE

(See Figure 13)

1. Deactivate all valves, cylinders and pumps.
2. Remove tape from spindles by continuous indexing.
3. Remove inlet lines to Stations # 1 and 2, and place in a suitable sump (beaker).
4. Detach nutrient (teflon tubing line) from tygon tubing.
5. Place tygon (nutrient source) line and sample source line in container of 4M Urea.
6. Activate Pumps # 1 and 2 for ~5 minutes.
7. Deactivate Pumps # 1 and 2.
8. Remove inlet lines to Stations 1 and 2, and relocate in 4M Urea container. Discard fluid in sump, and let these lines sit overnight.
9. Turn power off.
10. Turn air off.
11. Disconnect high voltage supply.
12. Place black cloth over PMT housing.

Appendix D

REAGENT PREPARATION

A. LUMINOL- H_2O_2 REAGENT

1. Luminol Solution

- a. 60.0 gms sodium hydroxide ("Baker Analyzed" Pellets Reagent) are dissolved in liter of filtered distilled water and allowed to cool to ambient temperature.
- b. 15.0 gms disodium ethylenedinitrilotetracetate dihydrate ("Baker Analyzed" Reagent Powder) are dissolved in above alkaline solution.
- c. Dissolve 1.00 gm of luminol (3-aminophthalhydrazide, Aldrich Chemical) in above. Let solution stand at ambient temperature overnight before use.
- d. The stock luminol solution may be stored in an unpigmented polyethylene or polypropylene container. The solution has a shelf life at ambient temperature of at least 6 months. Freezing does not affect the luminol solution, however, elevated temperatures accelerate the decomposition (with loss in sensitivity). For maximum shelf life, storage temperatures should not exceed 95°F.
- e. The usability of an aged luminol solution can be evaluated by determining the signal obtained with a fresh H_2O_2 and a fixed bacterial (E. coli) challenge. If the net signal is less than expected, the luminol would be suspect.

2. Hydrogen Peroxide Solution

- a. Dilute 3% hydrogen peroxide ("Baker Analyzed" Reagent) to 1% with filtered distilled water.
- b. The 1% H_2O_2 should be stored in an unpigmented polyethylene or polypropylene container and preferably at temperatures not exceeding 95°F (elevated temperatures accelerate decomposition). Current studies on shelf life of this reagent indicate at least one month stability at temperatures up to 37°C.

c. The efficacy of an aged hydrogen peroxide solution may be checked by the procedure outlined above (para. 1.e) for luminol. In actual use, the luminol, hydrogen peroxide and water (Premix diluent) are pumped (see Figure 13) through a glass mixing coil (4 ml holdup volume).

The respective flow rates are:

Luminol	0.60 ml/min.
1% H ₂ O ₂	1.10 ml/min.
H ₂ O	4.36 ml/min.

Buchler pumps (with tygon tubing) were used for metering the reagents; a variability of $\pm 10\%$ in the flow rates can be tolerated.

B. 4M UREA SOLUTION

Reagent grade ("Baker Analyzed") Urea is dissolved in glass-distilled water to a final concentration of 4 Molar. The solution is filtered through a 0.1 μ Ultripor filter and then passed through a mixed bed ion exchange column (equal quantities of Dowax AG 50W - X8 and Dowax AG1 - X8, 200-400 mesh, 200 ml bed volume). The pH of the effluent from the column is adjusted to pH 7.0 to 7.5 with hydrochloric acid and then filtered through a 0.2 μ (prewashed*) Millipore prior to use.

The 4M Urea may be stored in a glass, polyethylene or polypropylene container during use. The solution is stable for at least 7 weeks at temperatures up to 37°C.

To determine whether significant determination of a Urea solution has occurred on standing, the baseline signals obtained with the prescribed processing sequence are compared for aged and unaged Urea solutions. If significant decomposition has occurred, the aged Urea solution would be less effective in removing nutrient and so produce higher baseline values on reaction with luminol-H₂O₂.

* Pass about 500 ml of distilled water through a Millipore filter prior to use to remove finishing agent from filter.

C. DEXTROSE BROTH

1. Add 23.0 grams of Difco Dextrose broth to 1 liter of freshly distilled water.
2. Heat and stir until dissolution is complete.
3. Filter solution progressively through Nos. 3, 5 and 50 Whatman filters followed by filtration through a 0.8μ , 0.45μ and 0.22μ membrane filters.
4. Dispense into sterile glass bottles (100 ml volume) containing .031-inch I. D., 6-inch long teflon tubing used for extraction purposes when connected to pump. Secure bottle with foam cap.
5. Autoclave @ 15 psig for 15 minutes.
6. Remove and seal end of tubing.

D. PREPARATION OF BACTERIAL SUSPENSION

Bacteria (E. coli or S. marcescens) are 16 hour cultures grown on trypticase soy agar at 37°C . Bacteria are harvested by adding 3 to 4 ml of filtered distilled water to the Petri plate and rocking slowly to dislodge the bacteria from the agar. The bacterial suspension is then added to a centrifuge tube (15 ml) and filled to the three quarter mark with filtered distilled water. After centrifuging for 20 minutes at 5000 g, the supernatant is poured off and the tube again refilled with water to the same volume. After resuspending the pellet of sedimented bacteria by vortexing, the tube is again centrifuged as before. The supernatant is again poured off, the tube refilled with water and vortexed to resuspend the sedimented bacteria. A 0.1 ml aliquot of the bacterial suspension is added to 100 ml of filtered distilled water to make up the stock bacterial suspension. Final dilutions are made up in filtered distilled water.

"Total" (viable + non-viable) bacterial counts are made by direct microscopic count after prestaining the organism with an AMB protein-specific dye.

"Viable" counts are made at the time a sample is taken for processing of the viable cycle on the water monitor. The standard pour plate procedure is used for obtaining a viable count.